Small Molecules as Probes of Biological Systems

by

Poulami Talukder

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved January 2016 by the
Graduate Supervisory Committee:

Sidney M. Hecht, Chair
Neal Woodbury
Ian R. Gould

ARIZONA STATE UNIVERSITY

May 2016

ABSTRACT

The manipulation of biological targets using synthetic compounds has been the focal point of medicinal chemistry. The work described herein centers on the synthesis of organic small molecules that act either as probes for studying protein conformational changes or DNA–protein interaction, or as multifunctional radical quenchers.

Fluorescent labeling is of paramount importance to biological studies of proteins. For the development of new extrinsic small fluorophores, a series of tryptophan analogues has been designed and synthesized. Their pdCpA derivatives have been synthesized for tRNA activation and *in vitro* protein synthesis. The photophysical properties of the tryptophan (Trp) analogues have been examined, some of which can be selectively monitored even in the presence of multiple native tryptophan residues. Further, some of the Trp analogues form efficient FRET pairs with acceptors such as acridon-2-ylalanine (Acd) or L-(7-hydroxycoumarin-4-yl)ethylglycine (HCO) for the selective study of conformational changes in proteins.

Molecules which can bind with high sequence selectivity to a chosen target in a gene sequence are of interest for the development of gene therapy, diagnostic devices for genetic analysis, and as molecular tools for nucleic acid manipulations. Stereoselective synthesis of different alanyl nucleobase amino acids is described. Their pdCpA derivatives have been synthesized for tRNA activation and site-specific incorporation into the DNA-binding protein RRM1 of hnRNP LL. It is proposed that the nucleobase moieties in the protein may specifically recognize base sequence in the i-motif DNA through H-bonding and base-stacking interactions.

The mitochondrial respiratory chain accumulates more oxidative damage than any other organelle within the cell. Dysfunction of this organelle is believed to drive the progression of many diseases, thus mitochondria are an important potential drug target. Reactive oxygen species (ROS) are generated when electrons from the respiratory chain escape and interact with oxygen. ROS can react with proteins, lipids or DNA causing cell death. For the development of effective neuroprotective drugs, a series of *N*-hydroxy-4-pyridones have been designed and synthesized as CoQ_{10} analogues. All the analogues synthesized were evaluated for their ability to quench lipid peroxidation and reactive oxygen species (ROS).

ACKNOWLEDGMENTS

It has been a great experience to pursue my graduate studies under the supervision of Professor Sidney Hecht who has been an outstanding mentor over the last five years. I would like to thank him for his guidance, patience, continuous support and strict discipline. I would also like to take this opportunity to express my gratitude to my committee members Professor Ian Gould and Professor Neal Woodbury for their support and help. I would also like to thank my previous advisors Professor Tanmaya Pathak and Professor Srinivasan Chandrasekaran who have all educated and inspired me in the art of synthetic organic chemistry.

I thank the scientists in the Hecht laboratory who trained me as a chemist. They include Dr. Pablo Arce, Dr. Manikandadas Mathilakathu Madathil, Dr. Chandrabali Bhattacharya, Dr. Mohammad Parvez Alam, Sandipan Roy Chowdhury, Dr. Trevor Bozeman and Dr Ryan Nangreave for helping me numerous times with things related and unrelated to chemistry. I would like to acknowledge Prof. Shengxi Chen, Prof. Larisa Dedkova, Prof. Omar Khdour, Dr. Basab Roy, Dr. Petro Yakovchuk and Dr Sashka Daskalova, with whom I have collaborated. My thanks also go to all the current and former members of the Hecht group who have contributed to my research in their own ways. I would also like to give special thanks to dearest friends Dr. Mohammad Parvez Alam, Dr. Chandrabali Bhattacharya and Sandipan Roy Chowdhury for helping me and supporting me during every difficult time of my graduate life. I also want to extend my gratitude to my lab partners Dr. Sriloy Dey, Dr. Jose Armendariz and Indrajit Bandopadhyay for helping me through lab related issues. My thanks go to Gina Dunphy who has offered great help during my time in the Hecht lab.

It is also a pleasure to thank all my friends in Tempe who have all given their love, support, and encouragement during my time in Arizona. I would finally like to thank my family for their love, blessings and constant support. I am grateful for their patience and guidance and letting me follow my dreams. And finally, I would like to dedicate this thesis to my *amma* (paternal grandmother) and *dida* (maternal grandmother) for their unconditional love and blessings.

TABLE OF CONTENTS

Page

LIST OF ABBREVIATIONS

Acd	acridone-2-ylalanine
AcOH	acetic acid
Ac_2O	acetic anhydride
APCI	atmospheric pressure chemical ionization
anh	anhydrous
aq	aqueous
atm	atmosphere
BHT	butylated hydroxytoluene
Bn	benzyl
BnBr	benzyl bromide
Boc	*tert*-butoxycarbonyl
br s	broad singlet
^{o}C	degrees Celsius
^{13}C NMR	carbon nuclear magnetic resonance spectroscopy
CAN	cerric ammonium nitrate
cat	catalytic
CBz	carboxybenzyl
$CDCl_3$	deuterated chloroform
cm	centimeter
CNPhe	cyanophenylalanine
CoQ	coenzyme Q
conc	concentrated

Cs_2CO_3	caesium carbonate
CuCl	copper(I) chloride
δ	chemical shift (ppm)
d	doublet
dd	doublet of doublet
DBU	1,8-diazabiocyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DCC	N, N'-dicyclohexylcarbodiimide
DCF	2′,7′-dichlorofluorescein
DCFH-DA	dichlorodihydrofluorescein diacetate
DEM	diethyl maleate
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DIPEA	diisopropylethylamine
DMAP	dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic Acid
EMSA	electrophoretic mobility shift assay
ETC	electron transport chain
EtOAc	ethyl acetate
Et_3N	triethylamine
ESI	electrospray ionization

$FADH_2$	flavin adenine dinucleotide
FRDA	Friedreich's ataxia
g	gram(s)
GCMS	gas chromatography mass spectrometry
GSH	glutathione
h	hour(s)
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCO	L-(7-hydroxycoumarin-4-yl)ethylglycine
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
hnRNPLL	heterogeneous nuclear ribonucleoprotein L-like
HPLC	high-performance liquid chromatography
Hz	hertz
J	coupling constant
L	liter
m	multiplet
m	meta-chlorobenzoic acid
M	molar
M^+	molecular ion
MALDI-TOF	matrix assisted laser desorption ionization time of flight
mg	milligram(s)
μm	microgram(s)
MHz	mega Hertz

min	minutes
mL	milliliter
mM	millimolar
mmol	millimole(s)
MMT	monomethoxytrityl
MRQ	multifunctional radical quencher
μmol	micromole(s)
N	normal
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NBS	*N*-bromosuccinimide
NCS	*N*-chlorosuccinimide
NHS	*N*-hydroxysuccinimide
nm	nanometer
NMR	nuclear magnetic resonance
NVOCCl	4,5-dimethoxy-2-nitrobenzyl chloroformate
Pd/C	palladium-on-carbon
pdCpA	5′-*O*-phosphoryl-2′-deoxycytidylyl(3′→5′)adenosine
PMF	proton motive force
PPA	polyphosphoric acid
ppm	parts per million
*p*TsCl	*p*-toluenesulfonyl chloride
PTC	peptidyltransferase center

q	quartet
quin	quintet
R_f	ratio of fronts
RNA	ribonucleic acid
RRM	RNA recognition motif
ROS	reactive oxygen species
s	singlet
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMP	sub mitochondrial particles
t	triplet
TBA	tetrabutylammonium
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin Layer Chromatography
TMP	trimethoprim
TMSA	trimethylsilylacetylene
TOH	tocopherol
tRNA	transfer RNA
UV	ultraviolet
v	volume

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

LIST OF SCHEMES

Schemes Page

CHAPTER 1

INTRODUCTION

1.1. Protein Biosynthesis

Proteins are macromolecules comprised of amino acid residues that perform important biological functions. Proteins differ from one another most fundamentally in the sequences of their amino acids, which are dictated by the nucleotide sequences of their genes, and which usually result in a preferred specific three-dimensional structure. The folded protein structure determines the nature of the catalytic activity or structural function of the protein. Protein biosynthesis involves several events, including transcription of nuclear DNA to mRNA, and translation of mRNA with the stepwise incorporation of amino acids to afford proteins. Protein translation is mediated by the ribosome, which link amino acids together in the order specified by the messenger RNA (mRNA).[1] Ribosomes are comprised of two major components of unequal size (30S and 50S in *E. coli*). The small subunit decodes the RNA, while the large subunit mediates peptide bond formation.[2] Each subunit, composed of an array of ribosomal RNAs (rRNAs) and proteins, has three sites for binding tRNA, designated as the A-site (aminoacyl-tRNA site), P-site (peptidyl-tRNA site) and E-site (exit site for deacylated tRNA).[3] The 30S subunit contains a 16S rRNA and 21 different proteins, and 50S subunit consists of a 5S, 23S rRNA and 31 different proteins.[4]

Each tRNA recognizes an mRNA codon (contiguous set of three nucleotides), complementary to the tRNA anticodon, and carries an activated, cognate amino acid at its opposite end. Every cell contains at least 20 tRNA molecules, one for each naturally occurring amino acid, and each tRNA recognizes at least one codon (triplet sequence) in

the genetic code. There are 64 codons; 61 of them decode natural amino acids, while the other three are "nonsense" or stop codons which define the C-terminus of the growing polypeptide chain. mRNA translation proceeds through three distinct steps, namely initiation, elongation and termination. The process begins with activation of tRNA with its cognate amino acid by its aminoacyl-tRNA synthetase. Translation initiation involves the binding of the 30S ribosomal subunit to the mRNA and aminoacylated tRNA, with the 16S ribosomal RNA having base pairs complementary to the Shine–Dalgarno sequence in the bound mRNA.[4] Formation of the initiation complex requires initiation factors that are not permanently associated with the ribosome; these are designated as IF1, IF2 and IF3. Generally, the initiation codon translated is AUG, such that polypeptide synthesis starts with amino acid residue methionine (Met). In prokaryotes, the tRNA that starts translation is a methionine tRNA in which the Met residue is formylated ($tRNA_f^{Met}$). Only this tRNA is able to form the initiation complex and occupies the ribosomal P-site. Ribosomes elongate the polypeptide chains at a rate of 10 to 20 amino acids per second[4] with the help of nonribosomal proteins elongation factors (EF-Tu, EF-Ts and EF-G).[4] The elongation process starts with decoding where the ribosome selects and binds an aminoacyl-tRNA (bound to EF-Tu.GTP) whose anticodon is complementary to the mRNA codon in the A site.[5] The amino group of the aminoacyl-tRNA present in the A-site mediates a nucleophilic attack at the activated peptide of the peptidyl-tRNA ester in P-site, thereby forming a new peptide bond and transferring the nascent polypeptide to the tRNA in the A site (Figure 1.1). This event is followed by translocation of the tRNAs in the A- and P-sites to the P- and E-sites, respectively followed by dissociation of the deacylated tRNA from the E-site. Elongation factor EF-G

promotes translocation through GTP binding and hydrolysis. The next aminoacylated-tRNA, having an anticodon complementary to the next codon in the mRNA, is then bound to the vacant A-site. Elongation proceeds until termination is signaled by the presence of any one of the three mRNA stop codons namely UAG, UAA or UGA. In *E. coli*, protein release factors RF-1(recognizes UAA and UAG) or RF-2 (recognizes UAA and UGA) bind to the stop codon in the A-site and induce the transfer of the peptidyl group from the peptidyl-tRNA to water triggering the release of the nascent polypeptide and dissociation of ribosomal complex into 50S and 30S subunits.[5]

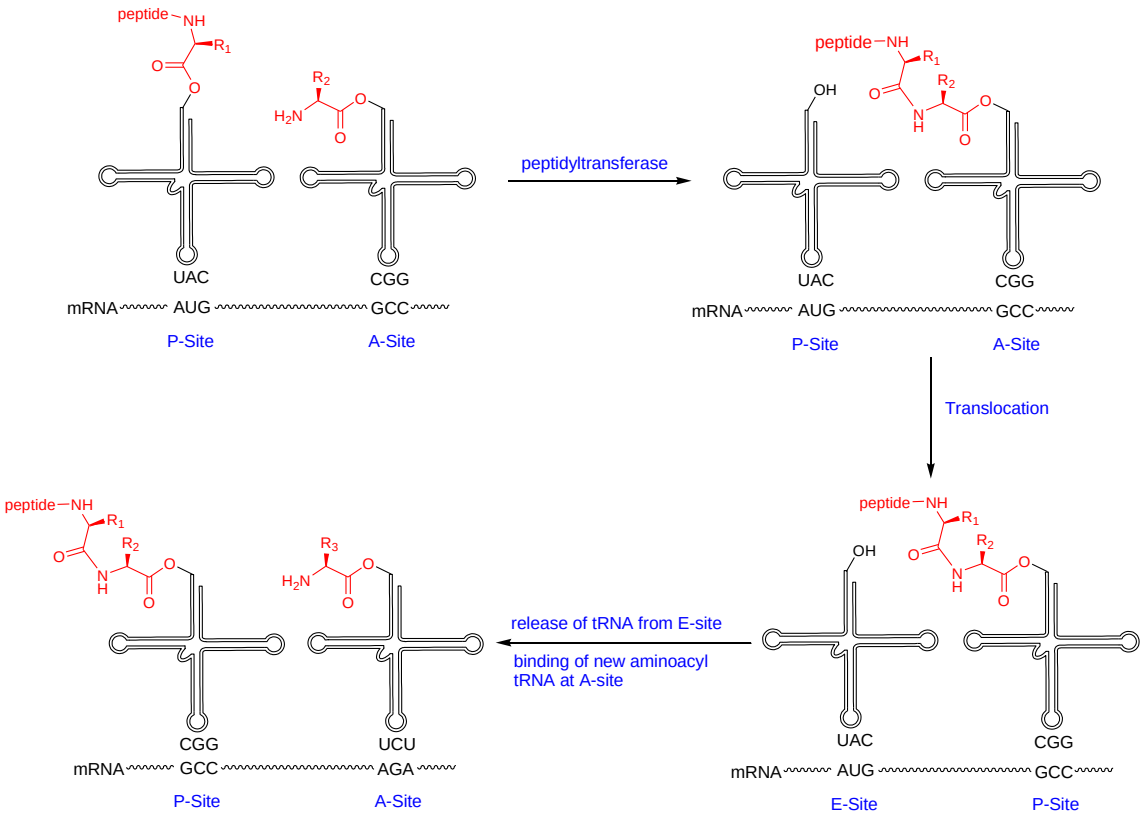


Figure 1.1. Peptide bond formation during protein translation where R_1, R_2 and R_3 are the side chain substituents of the amino acids.

1.2. Site-specific Incorporation of Unnatural Amino Acids into Proteins

The site-specific incorporation of noncanonical amino acids into proteins using misacylated suppressor tRNAs[6-9] has drawn significant attention due to its potential applications in studies of protein structure, function, dynamics and intermolecular interactions. This strategy, which involves site-directed mutagenesis of DNA to replace the codon for a specific amino acid of interest with a nonsense codon (Figure 1.2) has been developed into a very powerful tool to introduce a variety of new functionalities other than those found in the 20 proteinogenic amino acids.

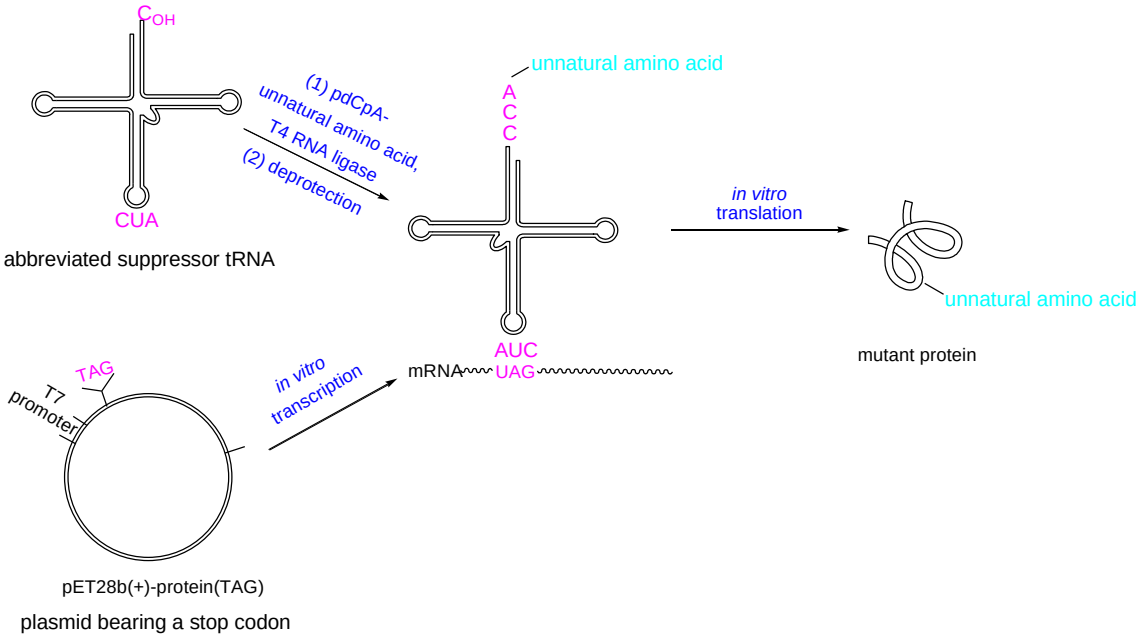
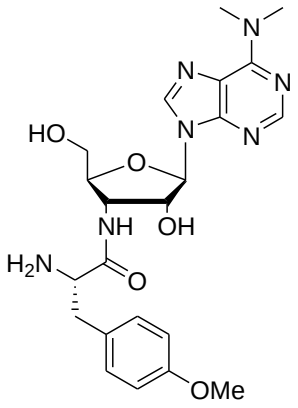


Figure 1.2. Strategy for the site-specific incorporation of unnatural amino acids into proteins *in vitro*.

A suppression event refers to suppressing a stop codon with a misacylated suppressor tRNA, thereby circumventing the effect of release factors that signal the termination of protein synthesis in the ribosome. The natural nonsense codons used for this purpose are UAG (amber),[6] UGA (opal)[10] and UAA (ochre)[11]. Hecht and co-workers

first developed this methodology by aminoacylating a dinucleotide, pCpA, with an N-protected amino acid and ligating the aminoacylated dinucleotide to a 74 nucleotide abbreviated tRNA (tRNA-C_{OH}) lacking the 3′-terminal cytidine and adenosine moieties, with the help of T4 RNA ligase.[12-14] Later, Schultz and co-workers modified the strategy by replacing the aminoacylated dinucleotide pCpA with dinucleotide pdCpA[15,16] which simplified the synthesis of the dinucleotide. This technology is now widely used for misacylation of tRNA, enabling the incorporation of a broad variety of unnatural amino acids into proteins.

More recently, Hecht and co-workers demonstrated that the modification of the 23S rRNA in bacteria can dramatically alter the architecture of the ribosomal peptidyltransferase center and allow the incorporation of D-amino acids, β-L-amino acids and dipeptidomimetics.[17-21] The selection of ribosomes having modifications in some regions of the 23S rRNA was done using puromycin derivatives. Puromycin[22,23] is an aminonucleoside antibiotic produced by *Streptomyces alboniger* (Figure 1.3). Puromycin is considered an aminoacyl-tRNA mimic and acts as a universal translation inhibitor. It binds to the A-site of the PTC and readily accepts the growing peptide chain from the peptidyl-tRNA in the P-site, which leads to premature chain release. Puromycin, and its derivatives having various amino acid side chains, show different chain termination efficiencies.[24,25]



1.1

Figure 1.3. Structure of the antibiotic puromycin.

1.3 Förster Resonance Energy Transfer (FRET) in Proteins

Fluorescence spectroscopy has become a crucial tool in biochemical research by virtue of its robustness and high sensitivity.[26] Fluorophores absorb light of a specific wavelength (λ_{ex}), and after a finite time (τ) (typically 1–10 nanoseconds), energy is emitted at a longer wavelength (λ_{em}). The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted to the number of photons absorbed, is a measure of the efficiency with which fluorescence processes occur. A broad variety of fluorescent molecules are currently available, which include protein fluorophores (e.g., green fluorescent protein), organic fluorophores (e.g., fluorescein, coumarins, cyanines, bodipy dyes) and even inorganic fluorescent nanoparticles (e.g., quantum dots).[26,27] The diversity in molecular and electronic structures has resulted in wide range of photophysical properties including molar absorptivity, Stokes shift, quantum yield, lifetime and also the ability to respond to their environment. The selection of a fluorophore mainly depends on the specific photophysical properties required, and the extent to which it can be integrated into a specific macromolecule without perturbing its

function. Green fluorescent protein (GFP) and its derivatives have found broad application in biochemistry and cell biology.[27] GFP was first isolated from jellyfish *Aequorea victoria* and can be encoded genetically as a fusion protein to specific proteins of interest. The use of GFP has some limitations due to its relatively large size (238 amino acids). Therefore, alternative chemoselective labeling techniques using low molecular weight compounds have been developed.[27] Fluorescent protein labeling by site-specific incorporation of unnatural amino acids is a useful alternative.[28-32]

Monitoring changes in the fluorescence spectrum of a protein is commonly used to detect conformational and electrostatic transitions due to ligand binding, protein–protein associations, or chemical transformations. The fluorescence signal can result from naturally occurring amino acids (e.g., tryptophan and tyrosine) or conjugated dyes.[33] Distance dependent phenomena, such as Förster resonance energy transfer (FRET), have facilitated the study of protein conformation and function by measuring changes in energy transfer efficiency between a donor and an acceptor.[34,35] The acceptor may be a fluorescent molecule, leading to longer wavelength emission, or a quencher.[35-39] The transfer process is effective when the donor and acceptor are separated by less than 10 nanometers. Size is important in FRET donor/acceptor pairs, as are their rotational degrees of freedom and propensity to interact with water. Typically, a FRET system in a protein may include two different variants of green fluorescent protein (GFP)[34,40-42] or two large polycyclic aromatic molecules.[43,44] The fluorophores/quenchers are often chemically tethered to proteins via flexible linkers.[43,44] The large size of a fluorophore can significantly perturb protein structure and function. This is particularly of concern when trying to monitor conformational changes in an enzyme active site. Moreover, the

flexible tethers provide the reporter molecules with still more conformational freedom, independent of actual protein conformational changes. Thus, to measure subtle conformational changes in proteins, it is important to develop smaller fluorophores /quenchers that can be efficiently incorporated into the normal peptide backbone.

A few studies have incorporated donor and acceptor amino acids into a protein, through decoding a four-base codon CGGG and a nonsense codon UAG in the presence of an an aminoacyl $tRNA_{CCCG}$ and an aminoacyl-$tRNA_{CUA}$,[45-48] to monitor protein backbone cleavage[45] and protein structural changes.[46-48] Dihydrofolate reductase (DHFR) bearing a fusion peptide at its N terminus was labeled with a fluorescent donor 7-azatryptophan (**1.2**) and acceptor dabcyl-1,2-diaminopropionic acid (**1.3**) which flanked an HIV-1 protease cleavage site (Figure 1.4).[27,45] Stoichiometric cleavage of the peptide by HIV-1 protease was monitored by FRET, with an increase in donor's fluorescence emission intensity quantitatively identical to that anticipated from protein cleavage (Figure 1.4).[27,45]

Hecht and co-workers used this strategy for studying conformational changes in DHFR. Two fluorescent amino acids, 4-biphenyl-L-phenylalanine (donor) and L-(7-hydroxycoumarin-4-yl)ethylglycine (acceptor) were incorporated into the protein, enabling a study of conformational changes associated with inhibitor (trimethoprim, TMP) binding (Figure 1.5).[48] The binding of TMP is known to produce only modest changes in DHFR structure, far smaller than those generally studied by FRET.[48] Thus, the incorporation of small fluorescent amino acids is a good strategy for monitoring of small conformational changes, such as those that occur during the catalytic cycle.[48]

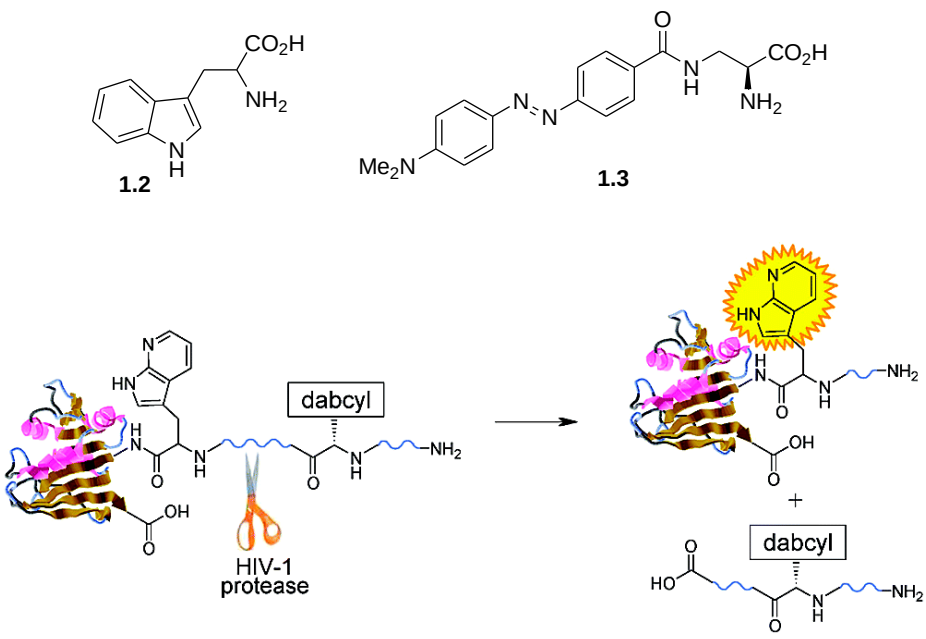


Figure 1.4. Cleavage of DHFR fusion protein containing donor (7-azatryptophan) and acceptor (dabcyl-1,2-diaminopropionic acid) by HIV-1 protease, resulting in a decrease in FRET and an increase in fluorescence emission intensity of the donor.[45]

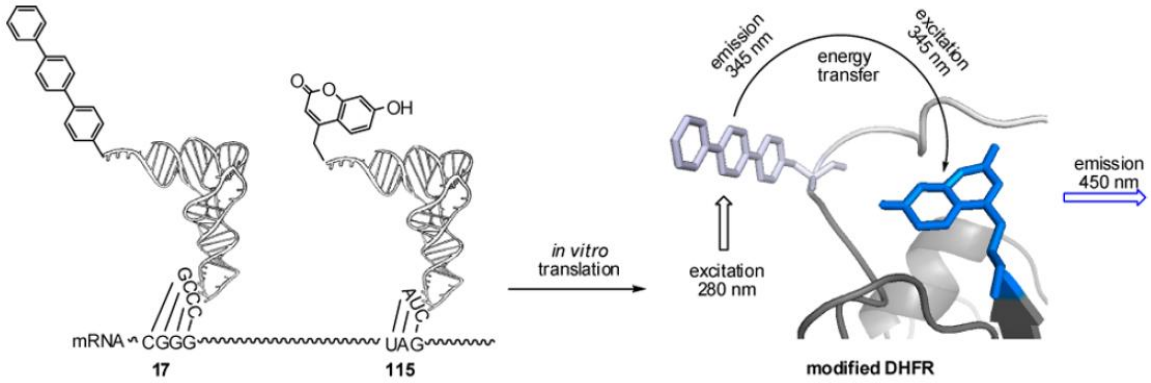


Figure 1.5. Strategy employed for incorporation of donor and acceptor amino acids into DHFR at positions 17 and 115, respectively.[48]

1.4. DNA–Protein Interaction

DNA–protein interaction plays an important role in the regulation of all aspects of DNA function including DNA replication, repair, recombination and transcription.[49]

DNA-binding proteins[50-52] contain DNA binding domains and may have affinity for either single or double stranded DNA. DNA-protein complexes[53] can be grouped into several classes, for example, polymerases that synthesize long chains or polymers of nucleic acids, nucleases which cleave DNA molecules, transcriptional factors which modulate the process of transcription, and structural proteins. Non-specific DNA interactions are formed through functional groups on the protein making ionic bonds to the acidic sugar-phosphate backbone of the DNA, and are largely independent of base sequence. In comparison, specific DNA-protein interactions depend largely on the base sequence and are stronger. Many sequence-specific DNA-binding proteins have been grouped into families.[54] Major families of motifs include the helix–turn–helix (HTH), homeodomain, helix, zinc fingers, zinc containing steroid receptors, leucine zipper and helix–loop–helix (HLH).[54-56] Many sequence-specific DNA-binding proteins are multimeric. Such multimeric interactions would be expected to increase the number of protein–DNA contacts, and thus to increase binding affinity.[54,57] However, there are monomeric sequence specific proteins as well, multimerization is not required for high affinity.[54] Most sequence-specific protein–DNA complexes have K_d values in the range of 10^{-11}-10^{-8} mol L^{-1}. Proteins with strict sequence specificity, generally show higher affinities *in vitro* (i.e. have lower K_d) than do proteins with a more 'relaxed' sequence specificity.[54]

The interactions between protein and nucleic acid are mediated by electrostatic attraction, hydrogen bonding and van der Waals contacts.[58] Electrostatic forces are long range, not very structure-specific, and contribute substantially to the overall free energy of association. Two other mechanisms (hydrogen bonding and van der Waals contacts)

are more sequence specific, especially hydrogen bonding interactions. This sequence specificity involves functional groups of the amino acids or backbone atoms of the protein and nucleobases in the grooves of the DNA (Figure 1.6).[54,59,60] Sequence-specific DNA-binding proteins generally interact with the major groove of B-DNA, because it

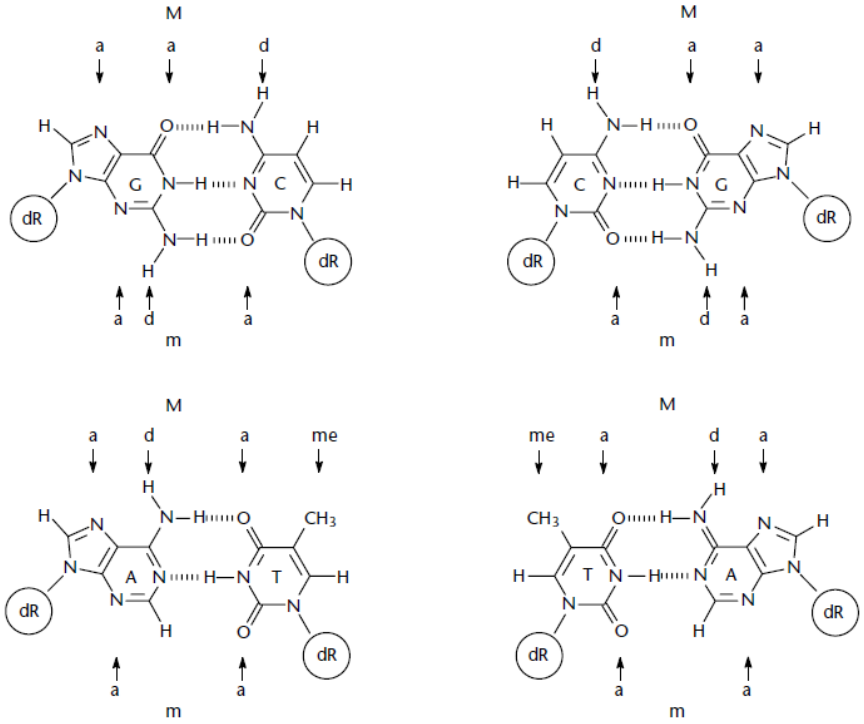


Figure 1.6. Points of recognition in the major (M) and minor (m) grooves of DNA for each of the four base pairs. a, electron acceptor; d, electron donor; me, methyl group. Hydrogen bonding in base pairs is indicated by dashed lines. dR in circles denotes the deoxyribose-phosphate backbone of DNA.[54]

exposes more functional groups.[59] Ninety percent of protein–nucleic acid hydrogen bonds involve a donor group on the protein and an acceptor group on the nucleic acid.[54,58] The protein backbone NH groups and the charged side chains of arginine, lysine, asparagine and glutamine are major hydrogen–bond donors. For DNA the phosphate oxygens and some atoms of guanine and adenine bases (N^7, O^6 for guanine and N^7, N^6 for adenine) are

the common acceptors for this type of interaction.[54,58] Adenine is mostly recognized by asparagine and glutamine whereas guanine is mostly recognized by lysine and arginine as donors (Figure 1.7).[54,58] Sometimes the hydrogen-bonding interactions between protein and DNA occur through water-mediated contacts.[61]

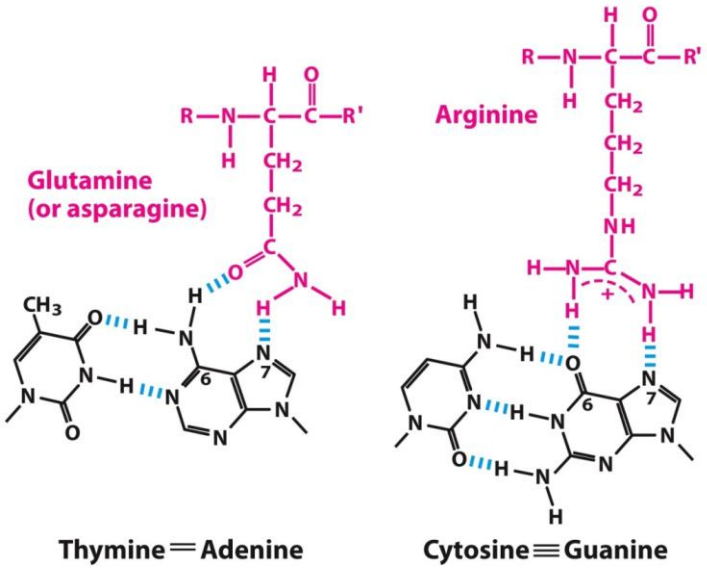


Figure 1.7. The H-bonding interaction between asparagine/glutamine and adenine, and between arginine and guanine.[63]

In addition to H-bonding involving the polar amino acids, nonpolar hydrophobic side chains such as alanine and isoleucine have been found to make van der Waals contact with the 5-methyl group of thymidine; the aromatic ring of phenylalanine can intercalate between base pairs in some complexes. Additionally, cation-π interactions[62] have also been found to be quite common at the interface between protein and DNA, and even more frequent than π-π stacking interactions. The majority of cation-π contacts involve adenines, and Arg-Ade is the most frequent cation-π pair.

1.5. Mitochondrial Respiratory Chain

Mitochondria are membrane enclosed organelles found in eukaryotic cells; they are responsible for important cellular functions such as energy metabolism, cell signaling and programmed cell death. Mitochondria generate ATP through the mitochondrial electron transport chain (ETC) coupled with oxidative phosphorylation (OX-PHOS). This process involves an pathway with five multisubunit protein complexes: Complexes I to IV (which constitute the electron transport chain) and ATP synthase (Complex V), and two free diffusible molecules. namely ubiquionone and cytochrome *c*. Electrons originating from NADH (Complex I) and $FADH_2$ (Complex II) pass from electron donors to electron acceptors via redox reactions throughout the ETC, and generate a proton (H^+) gradient, enabling the conversion of ADP to ATP in Complex V.[64-66]

The Krebs cycle provides the initial electron donors, NADH (nicotinamide adenine dinucleotide) and succinate. In functional mitochondria, the Complex I (NADH-ubiquinone oxidoreductase or NADH dehydrogenase) transfers two electrons from NADH to the lipid-soluble electron carrier ubiquinone (Q) or coenzyme Q (CoQ) (Figure 1.8). During this reaction, Complex I transfers 4 H^+ from the matrix to the intermembrane space for each two electrons received. Complex II (succinate dehydrogenase) also catalyzes the transfer of electrons from succinate (through $FADH_2$), to CoQ. However, unlike Complex I, no protons are transported to the intermembrane space in this pathway. Transfer of the first electron forms the free radical (semiquinone) form of Q and transfer of the second electron forms the semiquinone form to the ubiquinol form, QH_2. Reduced ubiquinol transfers two electrons to Complex III (cytochrome *bc1* complex or ubiquinol-cytochrome *c* oxidoreductase). Complex III transfers $2H^+$ from the matrix to

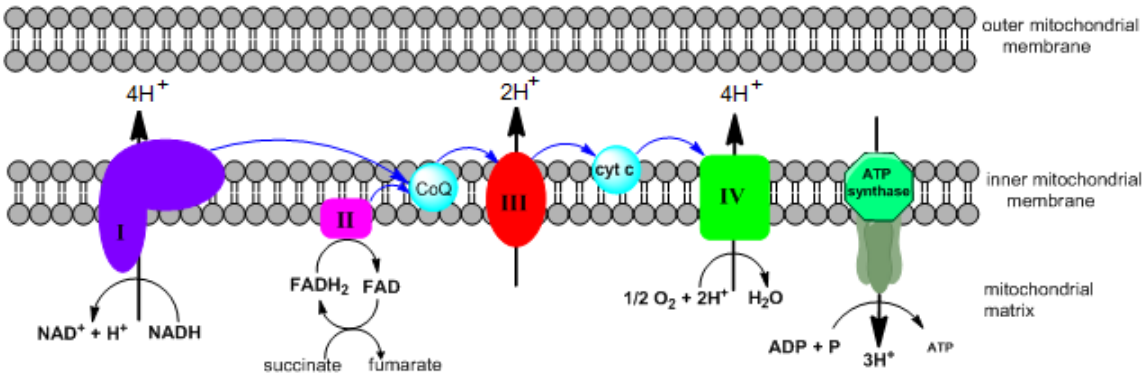


Figure 1.8. Mitochondrial membranes and different compartments involved in the electron transport chain (ETC).

intermembrane space and transfers two electrons from ubiquinol to cytochrome *c*. Cytochrome *c* serves as an electron carrier between Complex III and Complex IV (cytochrome *c* oxidase). Complex IV accumulates electrons from cytochrome *c* to reduce oxygen (the most electronegative and terminal electron acceptor in the chain), and transfer 4 H^+ protons into the intermembrane space. In summary, electrons are transported from the TCA cycle to oxygen, ultimately producing water along with the translocation of ten protons at Complex I ($4H^+$), Complex III ($2H^+$) and Complex IV ($4H^+$).[67,68]Although, some recent editions of the biochemistry textbooks mention a $4H^+/2e$, $4H^+/2e$, $2H^+/2e$ stoichiometry for complex I, III, and IV, respectively,[69,70] the analysis of chemical reactions occuring in ETC catalyzed by each complex, as well as thermodynamic considerations performed by Pardo and coworkers,[71]favors the stochiometry of 4, 2, 4 over 4, 4, 2 for Complexes I, III, and IV, respectively.

The proton motive force (PMF) generated by the translocation of ten protons from the matrix to intermembrane space by the electron transfer chain is the driving force for ATP synthase to synthesize ATP from ADP and inorganic phosphate (Pi).[72,73] A small

percentage of electrons leak directly from Complex I and Complex III to oxygen, resulting in the formation of the free radical superoxide (Figure 1.9A).[74-76] Complex I is the major source of superoxide in the respiratory chain. Although superoxide is not highly toxic,[77] it can undergo a spontaneous or superoxide dismutase catalyzed disproportionation reaction to form hydrogen peroxide (H_2O_2). In the presence of reduced iron or copper, peroxide can undergo the Fenton reaction[78,79] (Figure 1.9B) to produce highly toxic hydroxyl radicals, which diffuse through cells readily and are capable of reacting with virtually any biological molecule including DNA, proteins and lipids.[76,80]

Reactive oxygen species (ROS) are essential in living systems because of their role in many vital processes such as signal transduction and as a natural defense system against pathogens. ROS include free radicals, such as hydroxyl and superoxide radicals, which are substances with one or more orbital electrons with unpaired spin states, and nonradicals, including hydrogen peroxide and singlet oxygen.[76] Under normal conditions,

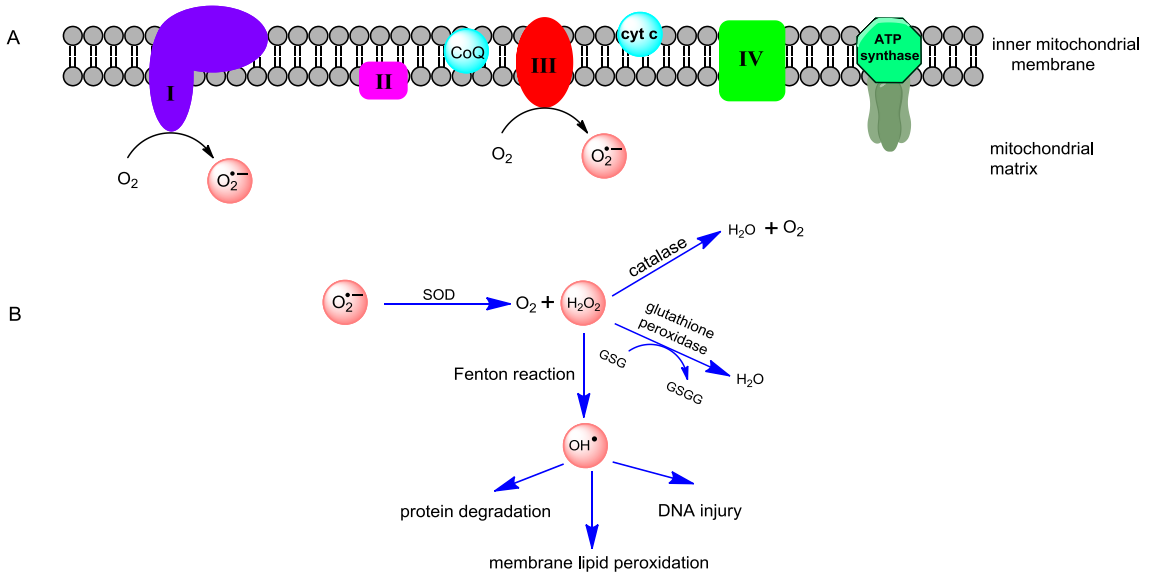


Figure 1.9. Mitochondrial formation and fate of superoxide (adapted from ref. 94).

ROS is maintained at a basal level by a network of low molecular weight antioxidants such as glutathione, α-tocopherol (vitamin E), L-ascorbic acid (vitamin C), and enzymes including glutathione peroxidase, superoxide dismutase (SOD) and catalase.[81-83] Given the role of mitochondria as the powerhouse of the cell, mitochondrial dysfunction is strongly linked to the pathogenesis of a number of human diseases (e.g. diabetes, cardiovascular diseases and neurodegenerative diseases).[84-91] Defects in any of the complexes in the electron transport chain can cause a mitochondrial disorder and can result in significant oxidative stress in the mitochondria with high mutation rates of mitochondrial DNA.[92-95] In neuronal degenerative diseases such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, the exposure to oxidative stress has been found to cause mutations in the mitochondrial DNA (mtDNA) causing mtDNA damage.[96-100] One of the primary targets of ROS is cellular lipids.[101-103] Abstraction of hydrogen atoms from lipid membranes forms a carbon centered radical. This radical can readily react with oxygen, forming a hydroperoxy radical (Figure 1.10). A lipid radical is again produced from the reaction of lipid with lipid peroxyl radical, thereby creating a chain reaction which can afford many lesions in the membrane lipids from a single hydroxyl radical.

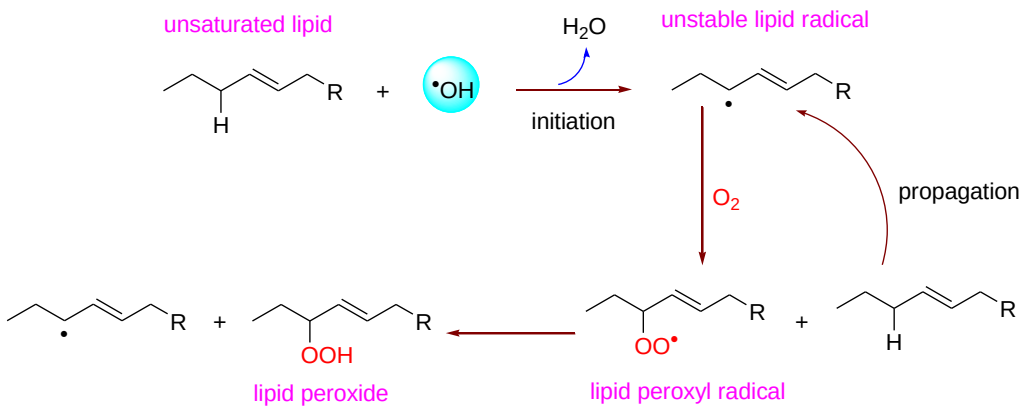


Figure 1.10: Radical chain reaction mechanism of lipid peroxidation.

The deleterious effects of oxidative stress are the reason that one of the major approaches for the treatment of mitochondrial disease involves appropriate antioxidants that can reduce the amount of intracellular ROS. It has been shown that a variety of naturally occurring antioxidants increase protection against oxidative stress in cells; vitamin E and CoQ_{10} therapy has been reported to be beneficial in patients with mitochondrial disease (e.g. Friedreich's ataxia (FRDA),[104-107] α-tocopherol (α-TOH) is among the most potent natural lipophilic antioxidants studied for quenching lipid peroxidation.[108-110] α-Tocopherol is able to transfer its phenolic H atom to a carbon-centered radical, and thereby quench the free radical chain reaction that occurs in the presence of O_2 (Figure 1.11).[111–113] The α-TOH radical generated is resonance stabilized, thus facilitating its formation associated with radical quenching.[114] α-TOH radicals can be converted back to α-TOH by interacting with the other redox molecules, notably NADH and vitamin C. Since the addition of oxygen to lipid radical to lipid peroxy radical (Figure 1.10) is extremely fast with rate constants on the order of 10^9 $M^{-1}s^{-1}$, the reaction of phenolic antioxidants with lipid radical is generally disregarded at their low

concentration. [115,116]

A problem in using these natural compounds as antioxidants is their low solubility in hydrophilic media such as extracellular and cytoplasmic fluids; this may limit their efficacy. One approach to obviate this problem has been to develop mitochondrially targeted ubiquinone analogues including the compounds MitoQ containing a lipophilic triphenylphosphonium cation.[117] Another approach has been to synthesize ubiquinone analogues with fewer carbons in the side chain compared to CoQ_{10} to facilitate exogeneous delivery.[118] A problem in using synthetic stoichiometric antioxidants such as BHT is a big excess amout would be needed to quech ROS.[119] Thus the identification of small molecule antioxidants which can potentially be used catalytically has become a critical goal.

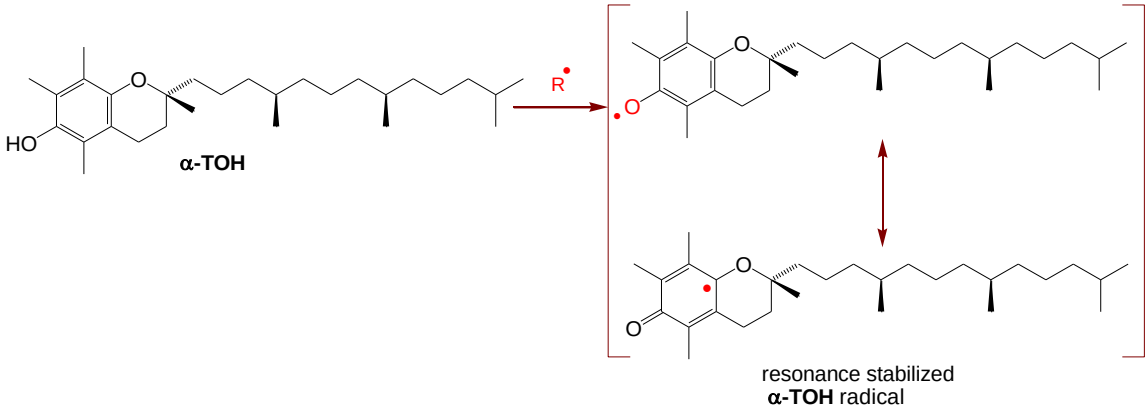


Figure 1.11. Quenching of radical-mediated lipid peroxidation by α-tocopherol where R· is a lipid radical.

CHAPTER 2

SYNTHESIS OF TRYPTOPHAN ANALOGUES FOR STUDYING PROTEIN CONFORMATIONAL CHANGES

2.1. Introduction

Tryptophan (Trp) is often important to protein function and structural integrity and is also the main source of intrinsic protein fluorescence.[120] Therefore, it has long been used as a probe of protein structure and function.[120] However, due to its complicated photophysics and multiple occurrence in some proteins of interest, Trp cannot always be used as a suitable optical probe.[121] Therefore, it seemed of interest to design novel tryptophan analogues having more useful spectroscopic properties and which do not perturb the local environment of the target proteins. Azatryptophans meet the above described criteria, where one of the endocyclic CH group of indole moiety is substituted with nitrogen. This substitution comprises not only the smallest possible structural alteration of all known Trp analogues but can also lead to substantial changes in the photophysical properties of the aromatic system. Among the azaindole chromophores, 4-azaindole has been identified as a promising candidate. It has strongly red-shifted fluorescence with a large Stokes shift (~130 nm). It is also biocompatible, as its 4-azatryptophan derivative can be incorporated into target proteins. However, the fluorescence intensity and quantum yield of 4-azatryptophan are much lower than that of tryptophan itself. In addition, azatryptophans are hydrophilic, such that their presence in the hydrophobic core of proteins could modify protein structure and function. To overcome these limitations we have prepared the more hydrophobic *N*-Me-4-azatryptophan and also two tricyclic 4-azatryptophan derivatives (Figure 2.1) having

larger Stokes shifts (~150-160 nm) and significantly higher molar absorptivities than that of tryptophan.

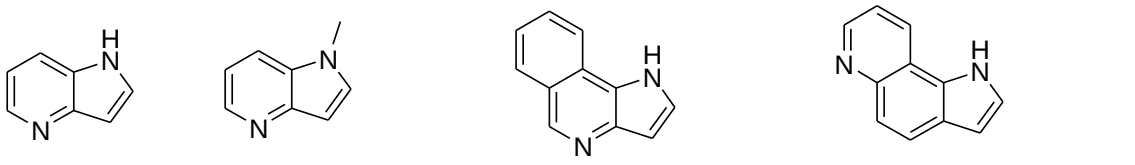


Figure 2.1. Structure of 4-azaindole and the chromophoric moieties of the tryptophan analogues. 1*H*-pyrrolo[3,2-*c*]isoquinoline consists of a benzene ring fused to a bicyclic 4-azaindole ring and 1*H*-pyrrolo[2,3-*f*]quinoline is a 4-azaindole modified with a benzo ring spacer, separating the pyridine ring from the pyrrole ring.

7-Azatryptophan is a another promising alternative to tryptophan as a photophysical probe as its chromophoric moiety, 7-azaindole, has absorption and emission spectra red shifted by 10 and 46 nm, respectively, from those of tryptophan.[120,122] *N*-Me-7-azaindole[122] (Figure 2.2) has been found to have better fluorescence properties than 7-azaindole itself because it retains the pronounced red shift present in the parent 7-azaindole, but with stronger fluorescence, prompting us to study this analogue.

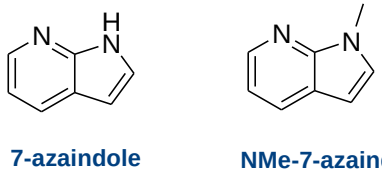


Figure 2.2. Structures of 7-azaindole and *N*Me-7-azaindole.

We also have investigated three different cyanotryptophan (CNTrp) analogues as potential probes for fluorescence analysis of protein structure and function. Recently, *p*-cyanophenylalanine (CNPhe)[123] has emerged as a useful spectroscopic probe for studying protein structure and dynamics. Introduction of the cyano group increased the

fluorescence quantum yield of CNPhe in water to about five times that of phenylalanine (Phe). The polarity of the cyano group is intermediate between that of a methylene and an amide group. This intermediate polarity allows CNPhe to be accepted in both hydrophobic and hydrophilic environments in a protein.[124] These interesting properties of CNPhe encouraged us to explore the fluorescence properties of cyanotryptophans. Thus far, only 5-cyanotryptophan has been used as an infrared probe of the local hydration status of proteins or peptides but its fluorescence quantum yield is approximately one order of magnitude smaller than that of Trp, rendering it less useful as a fluorescence probe.[125] Therefore, three cyanotryptophans were synthesized along with six azatryptophans (Figure 2.3).

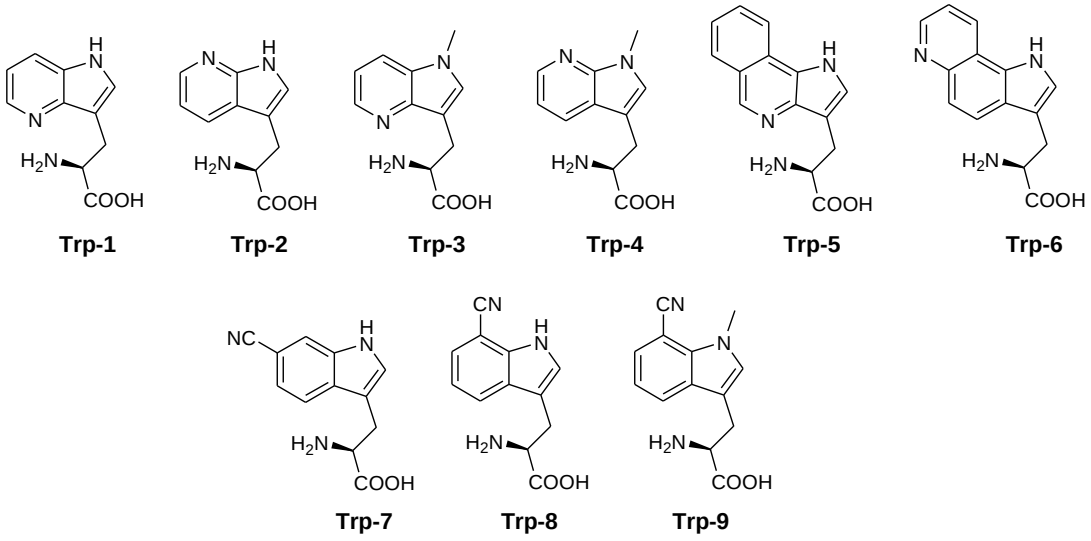


Figure 2.3. Tryptophan analogues (**Trp-1** – **Trp-9**) which were synthesized and incorporated into different positions of dihydrofolate reductase (DHFR).

To realize the asymmetric synthesis of the Trp analogues, a stereoselective strategy utilizing the Schöllkopf chiral reagent has been adopted.[126] This strategy has

enabled the successful synthesis of non-proteinogenic *S*-amino acids. In order to evaluate the properties of the Trp analogues as constituents of DHFR, their aminoacylated pdCpA (Figure 2.4) were synthesized, employed for the preparation of misacylated tRNAs, and then they were introduced site-specifically into DHFR.

DHFR is an essential and ubiquitous enzyme required for normal cellular metabolism in both prokaryotes and eukaryotes. The main role of DHFR is maintenance of the intracellular pools of 5,6,7,8-tetrahydrofolate, which is required for the biosynthesis of purines, pyrimidines and several amino acids.[127] DHFR catalyzes formation of 5,6,7,8-tetrahydrofolate from 7,8-dihydrofolate using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor (Figure 2.5).[127]

As discussed in Chapter 1, few studies have incorporated donor and acceptor amino acids into a single protein. Using misacylated suppressor tRNAs with two different anticodons, a Trp donor and an acceptor amino acid acridone-2-ylalanine (**Acd**)[128-130] were both incorporated into DHFR. Therefore, the aminoacylated pdCpA derivative of amino acid **Acd** was also synthesized (Figure 2.6).

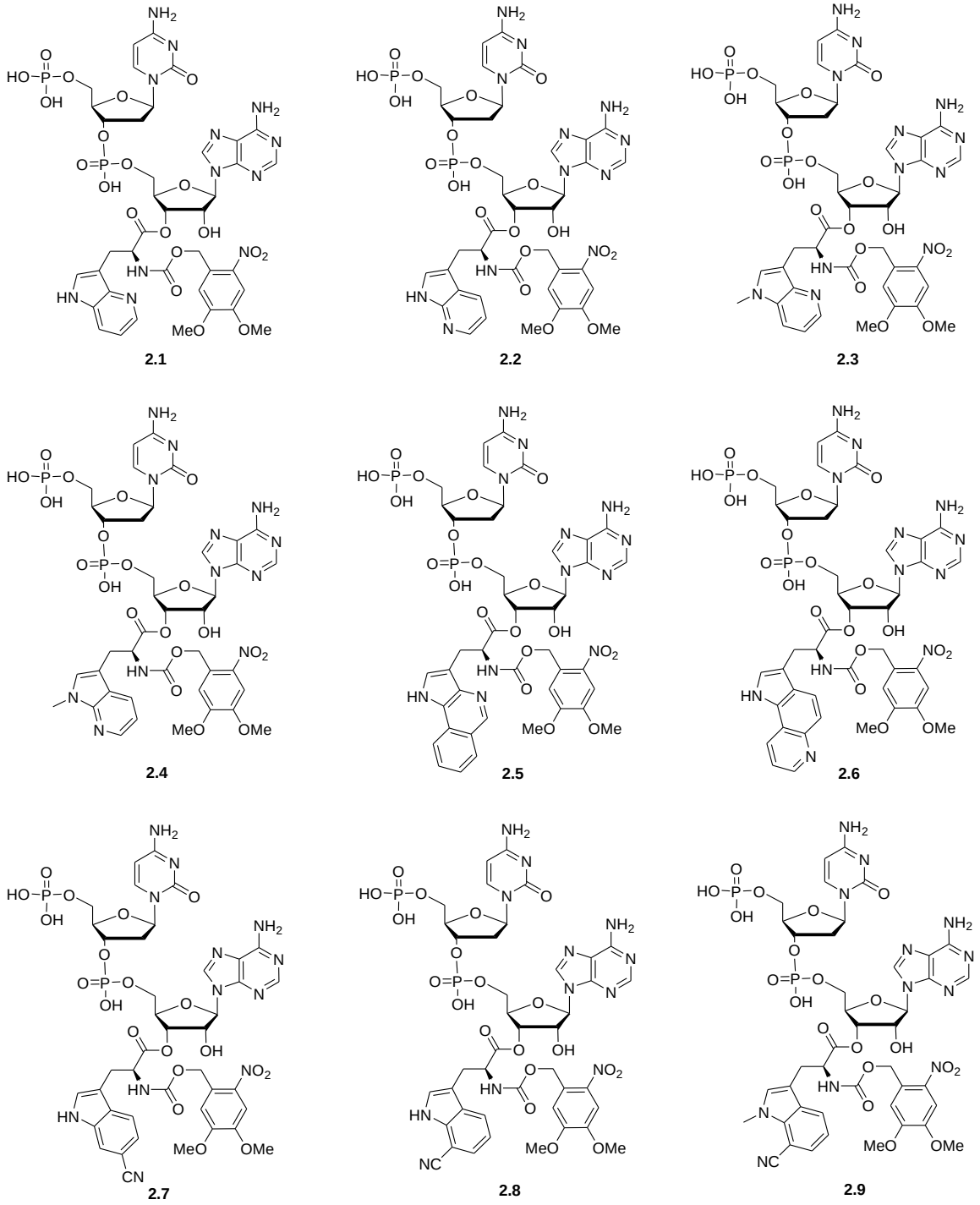


Figure 2.4. Series of aminoacylated pdCpA derivatives synthesized for site-directed incorporation of modified Trps at different positions of DHFR.

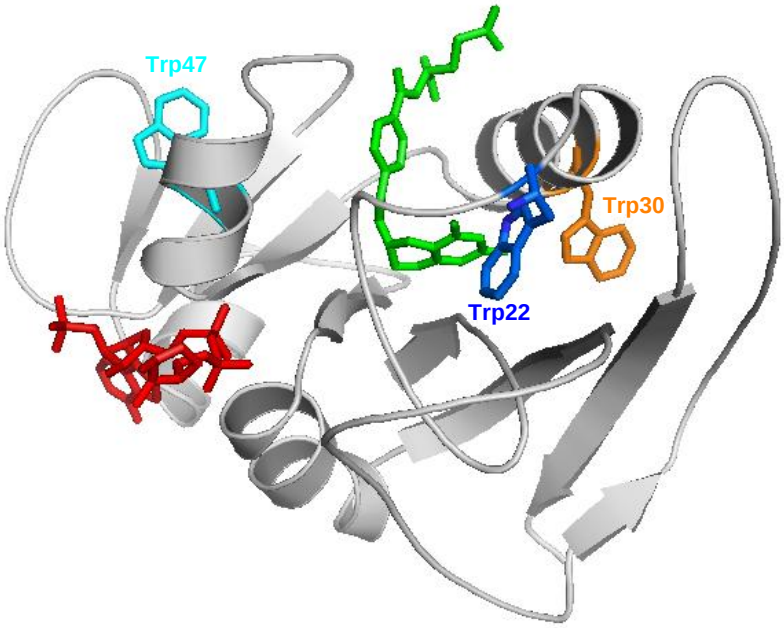


Figure 2.5. Structure of wild-type *E. coli* DHFR (PBD entry 1RX6), including Trp22, Trp30, and Trp47. The substrate tetrahydrofolate is shown in green and the cofactor NADPH is shown in red.

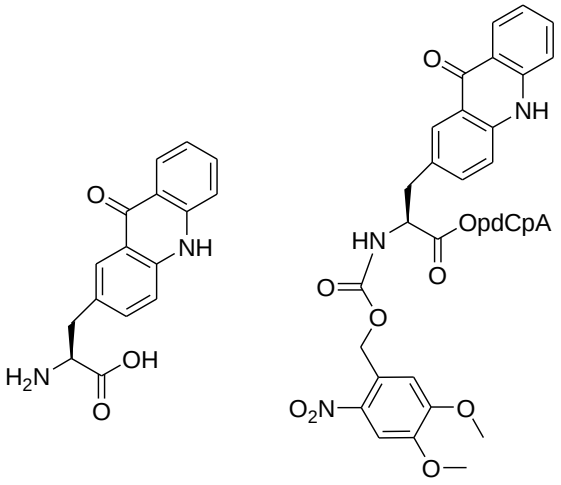


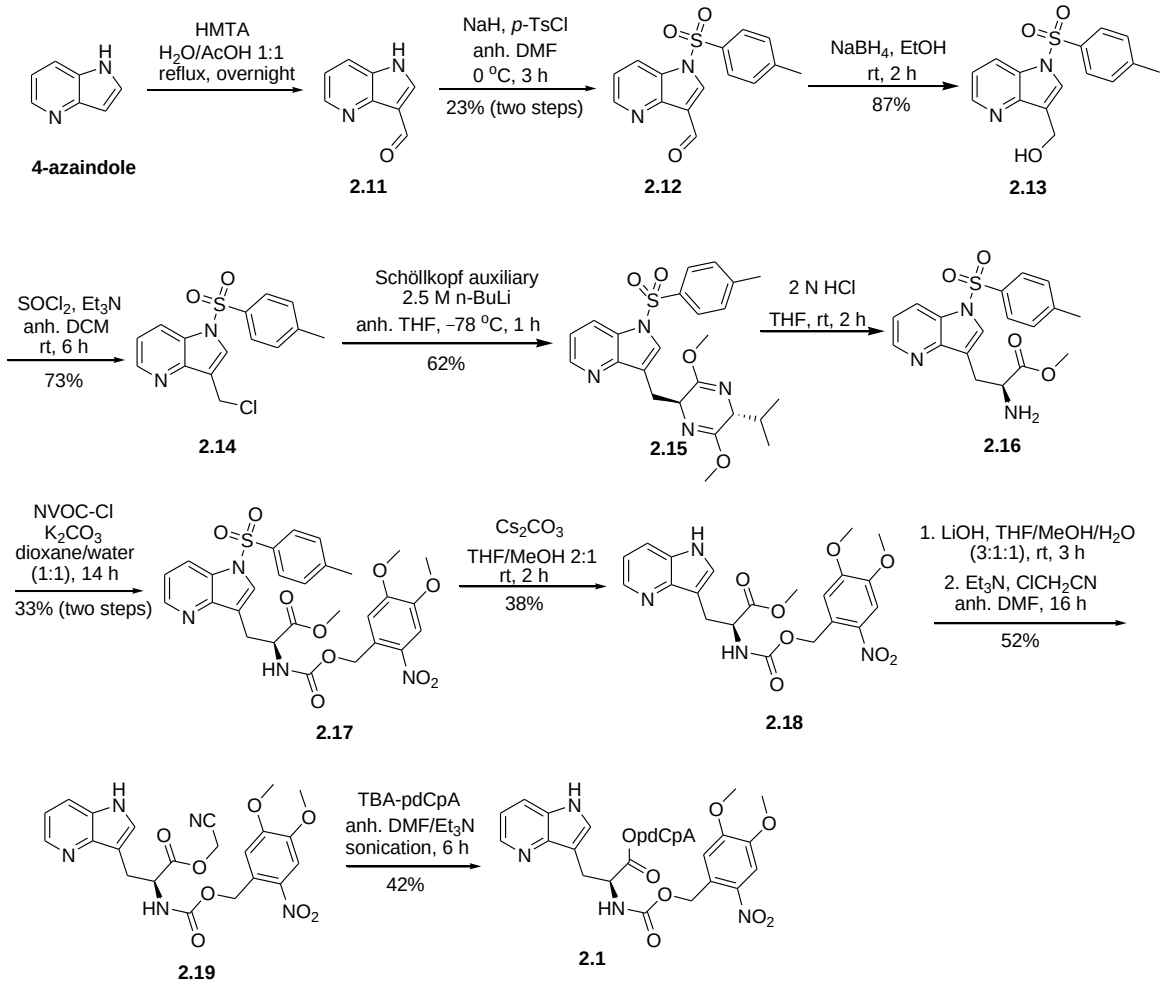
Figure 2.6. Structures of acceptor amino acid acridone-2-ylalanine (**Acd**) and its pdCpA derivative synthesized for tRNA activation and site-directed incorporation into DHFR.

2.2. Results

Synthesis of Fluorescent Tryptophanyl pdCpAs Esters

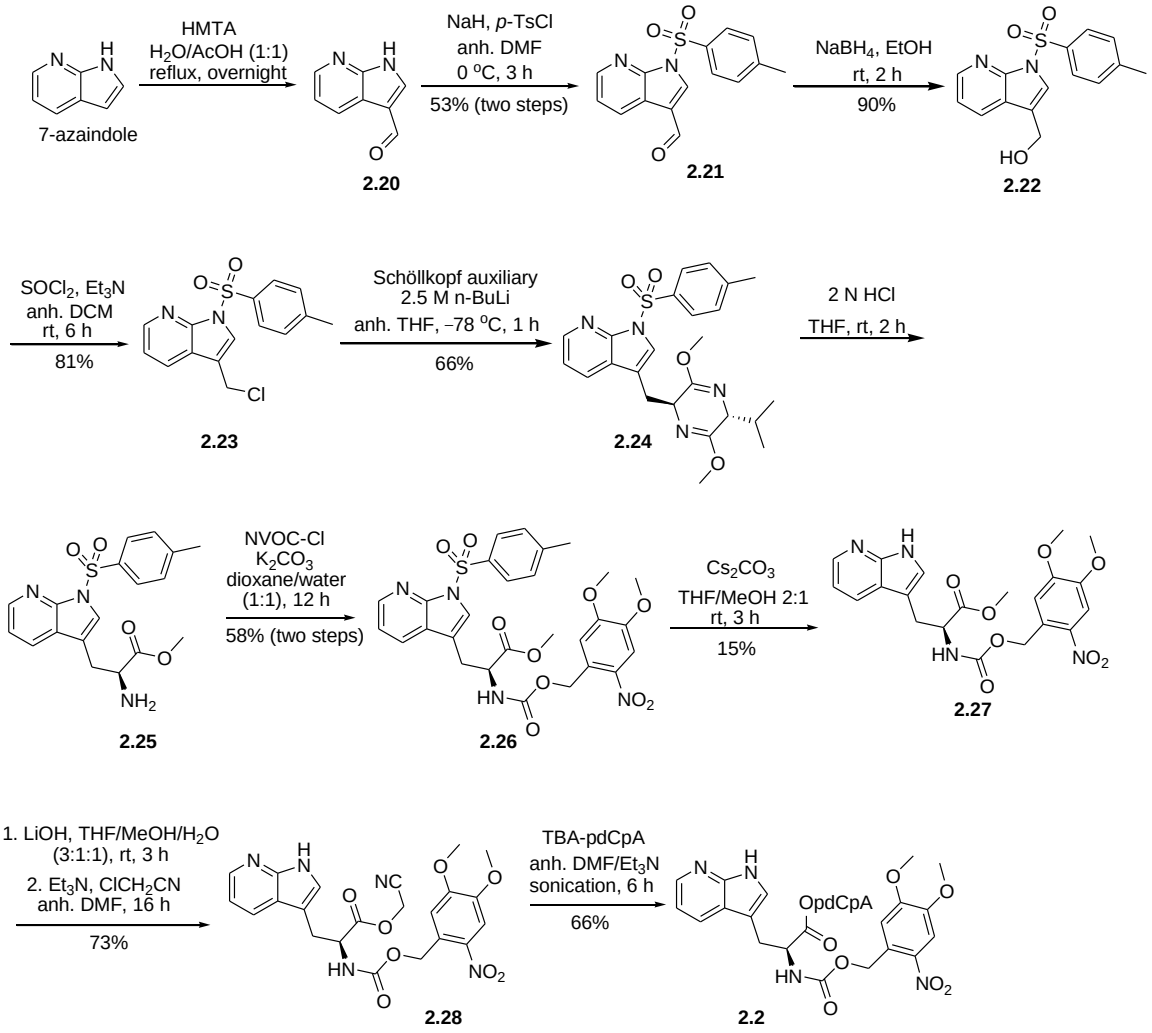
The synthesis of the aminoacylated pdCpA derivative of amino acid **Trp-1** (Scheme 2.1) was accomplished starting from commercially available 4-azaindole. This compound was formylated to yield **2.11** which was *N*-tosylated with *p*-TsCl and NaH, affording **2.12** in 23% overall yield.[131,132] Reduction of aldehyde **2.12** with $NaBH_4$ in EtOH afforded alcohol **2.13** in 87% yield.[131] Chlorination of **2.13** with thionyl chloride then provided **2.14**.[133] Asymmetric synthesis of the amino acid precursor was carried out using the Schöllkopf reagent ((*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine).[126] Regioselective lithiation (*n*-butyllithium, THF, ⁻78°C) of the Schöllkopf reagent produced the lithium enolate which afforded adduct **2.15** in 62% yield with high diastereoselectivity.[126] Mild hydrolysis (2 N HCl) provided α-substituted amino acid methyl ester **2.16** which was protected as the *N*VOC carbamate to yield **2.17** in 33% overall yield.[48] *N*-detosylation of **2.17** using cesium carbonate in 2:1 THF–MeOH afforded ester **2.18**.[134] Hydrolysis of **2.18** then afforded the free acid, which was activated as cyanomethyl ester **2.19** in 52% overall yield. Treatment of cyanomethyl ester **2.19** with the tris(tetrabutylammonium) salt of pdCpA[15] in anhydrous DMF gave the aminoacylated pdCpA containing amino acid **Trp-1** in 42% yield.

The synthesis of the aminoacylated pdCpA derivative of amino acid **Trp-2** (Scheme 2.2) was accomplished starting from commercially available 7-azaindole. Formylation of 7-azaindole under Duff reaction conditions[131] yielded aldehyde **2.20**, the latter of which was subjected to *N*-tosylation with *p*-toluenesulfonyl chloride (*p*-TsCl)



Scheme 2.1. Synthesis of **Trp-1** and its Aminoacyl-pdCpA.

and sodium hydride to afford **2.21** in 53% overall yield (Scheme 2.2). Reduction of aldehyde **2.21** to alcohol **2.22** using $NaBH_4$ in EtOH proceeded almost quantitatively. Chlorination of alcohol **2.22** with thionyl chloride afforded **2.23** in 81% yield. Regioselective lithiation (*n*-BuLi, THF, –78 °C) of the chiral auxiliary produced the lithium enolate, which afforded the adduct **2.24** from **2.23** with high diastereoselectivity. Mild hydrolysis (2 N HCl) provided *α*-substituted amino acid methyl ester **2.25** which was protected as the *N*VOC carbamate to yield **2.26** in 58% overall yield. *N*-Detosylation

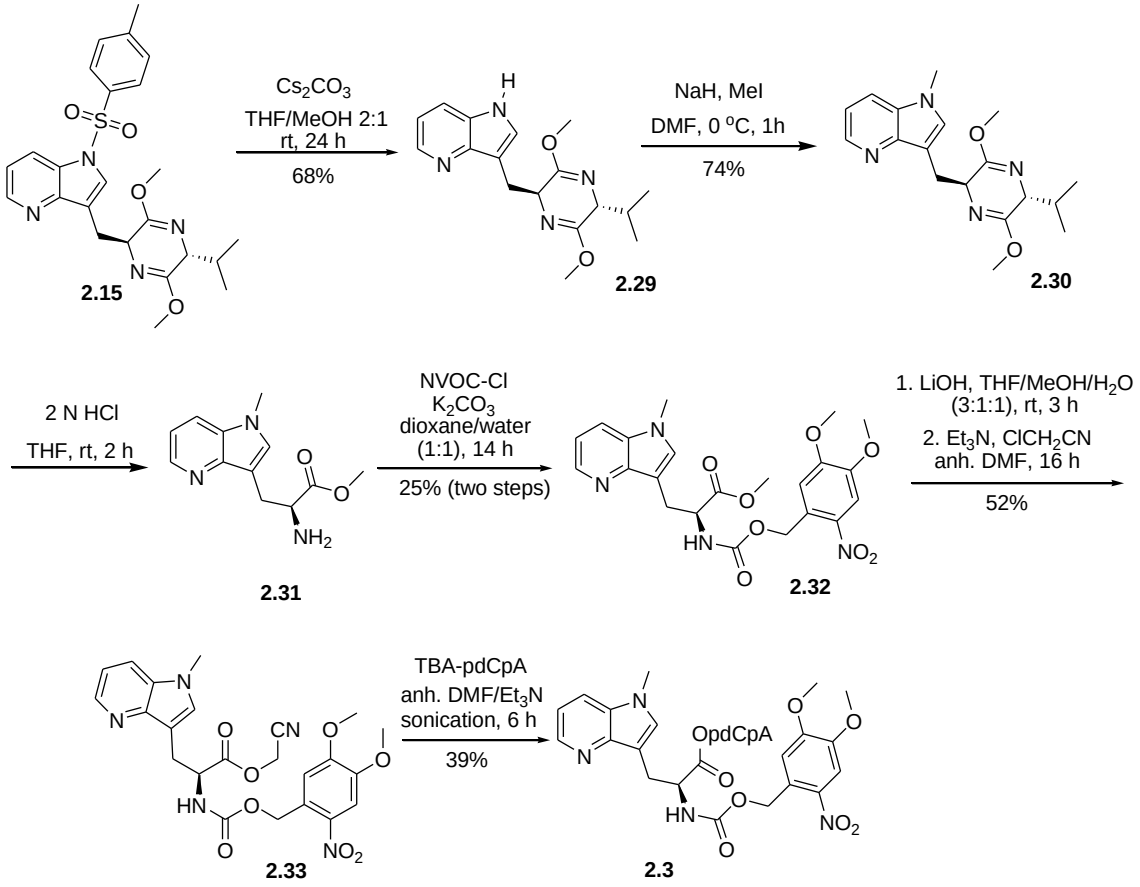


Scheme 2.2. Synthesis of **Trp-2** and its Aminoacyl-pdCpA.

of **2.26** using cesium carbonate in 2:1 THF–MeOH afforded ester **2.27**. Hydrolysis of **2.27** then afforded the free acid, which was activated as cyanomethyl ester **2.28** in 73% overall yield. Treatment of cyanomethyl ester **2.28** with the tris(tetrabutylammonium) salt of pdCpA in anhydrous DMF gave the aminoacylated pdCpA containing amino acid **Trp-2** in 66% yield (Scheme 2.2).

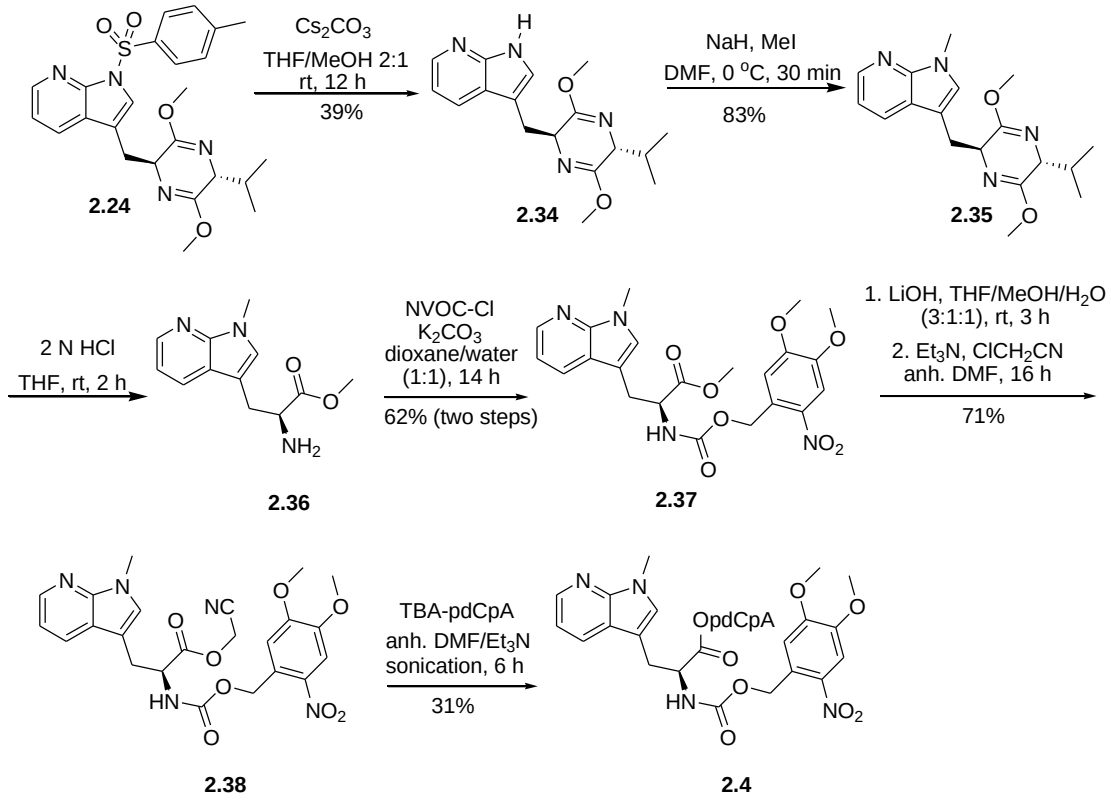
For the synthesis of the aminoacylated pdCpA derivative of amino acid **Trp-3** (Scheme 2.3), *N*-detosylation of **2.15** was performed using cesium carbonate in 2:1 THF–

MeOH to yield **2.29** in 68% yield. *N*-methylation of **2.29** with methyl iodide and sodium hydride afforded **2.30** in 74% yield.[132] Mild hydrolysis (2 N HCl) afforded the α-substituted amino acid methyl ester **2.31** which was protected as the *N*VOC carbamate to yield **2.32** in 25% overall yield. Methyl ester **2.32** was then hydrolyzed to afford the free acid which was subsequently treated with chloroacetonitrile to afford the requisite cyanomethyl ester **2.33** in 52% yield. Treatment of the cyanomethyl ester with a solution of the tris(tetrabutylammonium) salt of pdCpA in dry DMF gave the aminoacylated pdCpA containing amino acid **Trp-3** in 39% yield.



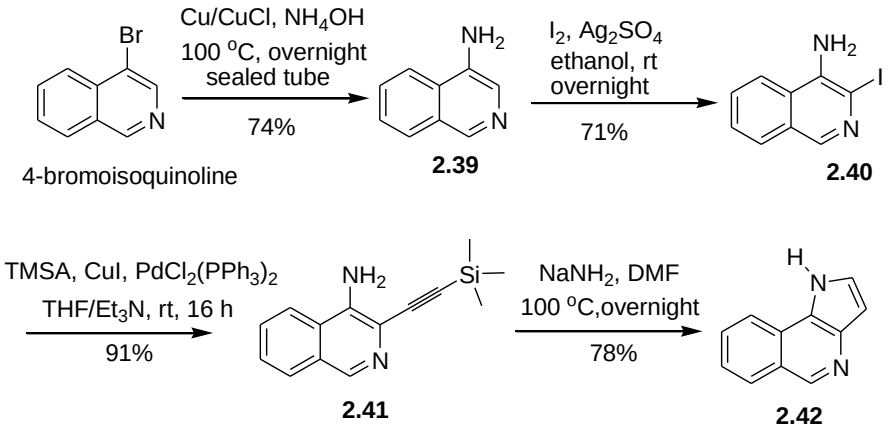
Scheme 2.3. Synthesis of **Trp-3** and its Aminoacyl-pdCpA.

For the synthesis of the aminoacylated pdCpA derivative of amino acid **Trp-4** (Scheme 2.4), *N*-detosylation of **2.24** was performed using cesium carbonate in 2:1 THF–MeOH to yield **2.34** in 39% yield. *N*-methylation of **2.34** with methyl iodide and sodium hydride afforded **2.35** in 83% yield. Mild hydrolysis (2 N HCl) of **2.35** afforded the α-substituted amino acid methyl ester **2.36** which was protected as the *N*VOC carbamate to yield **2.37** in 62% overall yield. Methyl ester **2.37** was then hydrolyzed to afford the free acid which was subsequently treated with chloroacetonitrile to afford the requisite cyanomethyl ester **2.38** in 71% yield. Treatment of cyanomethyl ester **2.38** with the tris(tetrabutylammonium) salt of pdCpA in anhydrous DMF afforded pdCpA ester **2.4** in 31% yield.

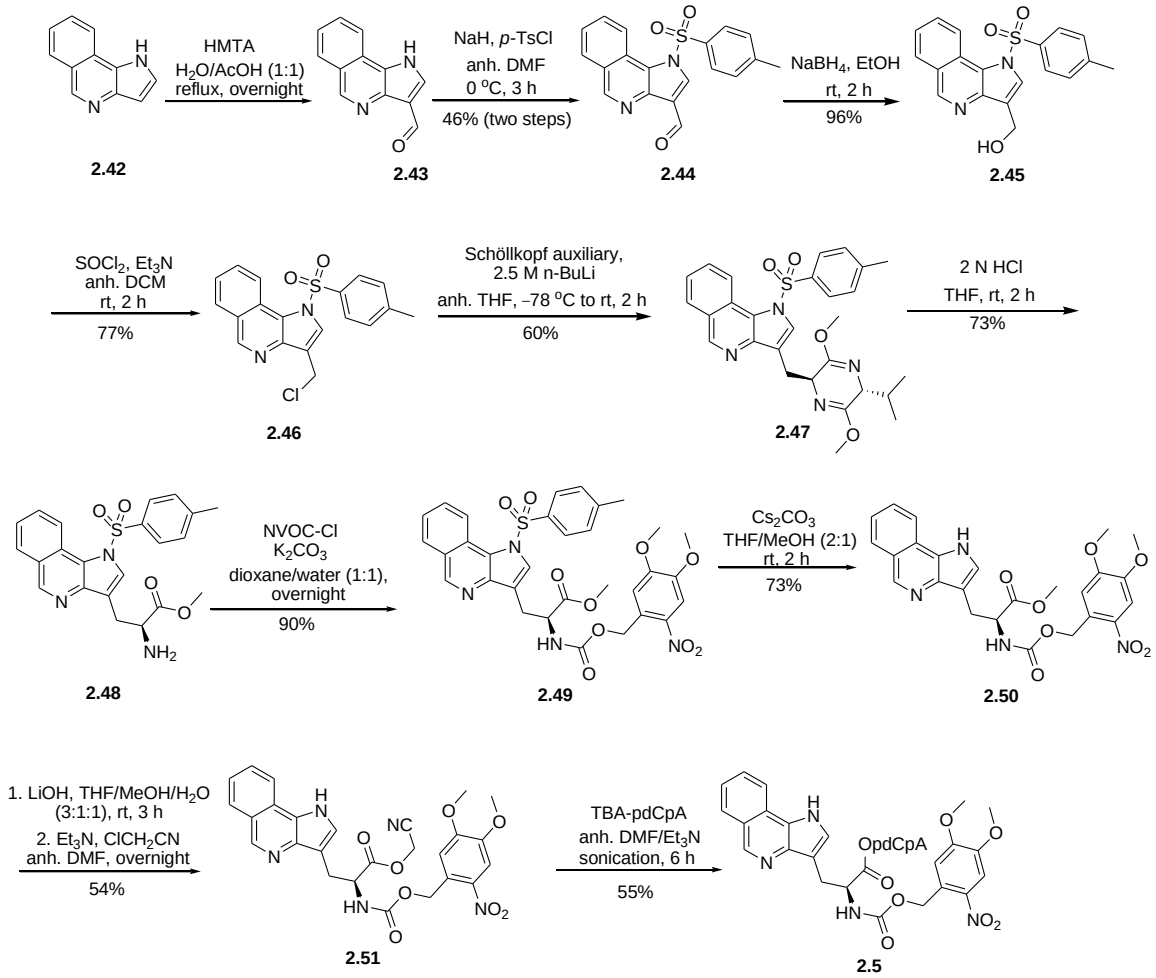


Scheme 2.4. Synthesis of **Trp-4** and its Aminoacyl-pdCpA.

For the asymmetric synthesis of **Trp-5**, the required indole was prepared in four steps (Scheme 2.5) from commercially available 4-bromoisoquinoline according to the method of Dupas et al.[135] with some modifications. The synthesis began with amination of 4-bromoisoquinoline (Scheme 2.5) using Cu/CuCl couple to afford **2.39**.[136] Iodination with iodine/silver sulfate system provided 4-amino-3-iodoisoquinoline (**2.40**) in 71% yield.[137] Sonogashira coupling of trimethylsilylacetylene (TMSA) and 4-amino-3-iodoisoquinoline (**2.40**) proceeded very smoothly at room temperature; the desired ethynyl derivative **2.41**[135] was obtained in 91% yield. The aminoethynyl derivative **2.41** was then heated to reflux with $NaNH_2$ in DMF to effect cyclization into key intermediate **2.42**[135] which was formylated to yield aldehyde **2.43** (Scheme 2.6). This intermediate was subjected to *N*-tosylation with *p*-toluenesulfonyl chloride (*p*TsCl) and sodium hydride to



Scheme 2.5. Synthesis of Pyrroloisoquinoline **2.42**.

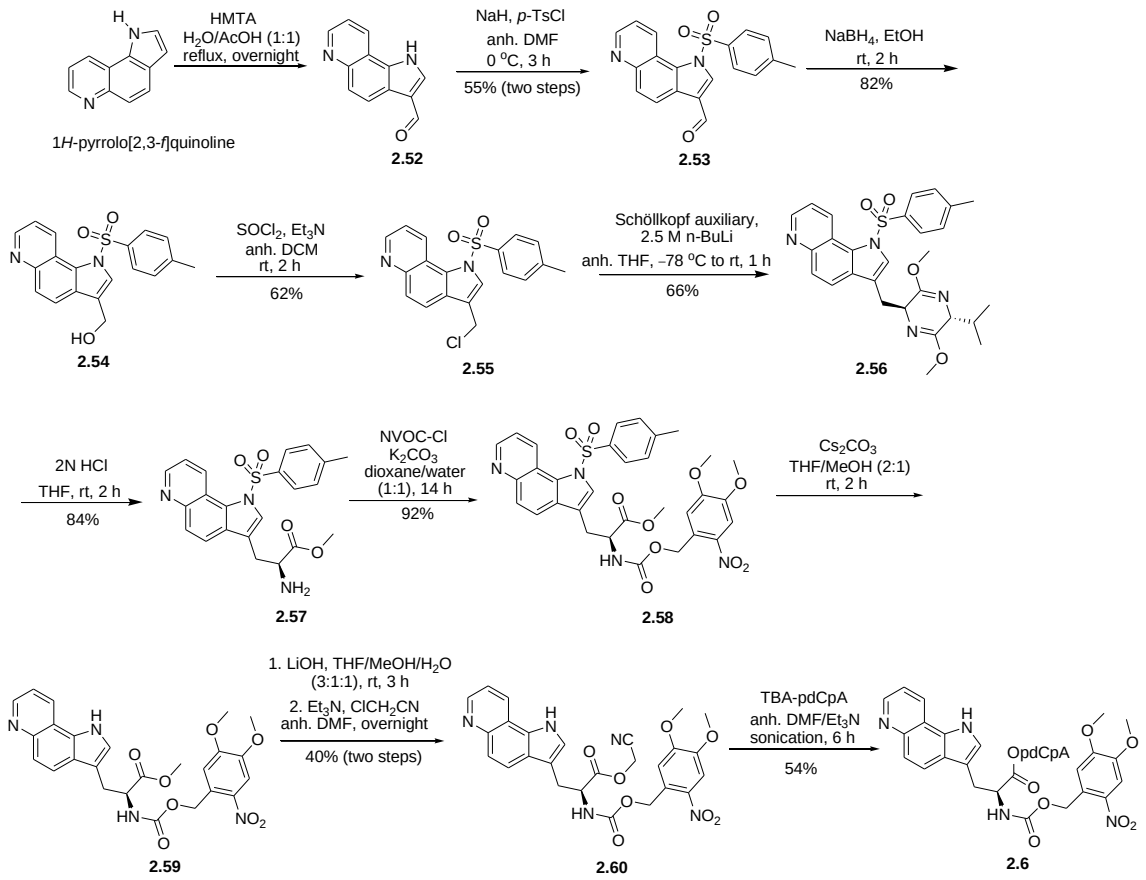


Scheme 2.6. Synthesis of **Trp-5** and its Aminoacyl-pdCpA.

afford **2.44** in 46% overall yield. Reduction of aldehyde **2.44** to alcohol **2.45** using $NaBH_4$ in EtOH proceeded almost quantitatively. Chlorination of alcohol **2.45** with thionyl chloride afforded **2.46** in 77% yield. Asymmetric synthesis of the amino acid precursor was carried out using the Schöllkopf reagent, (*R*)-2,5-dihydro-3,6-dimethoxy-2- isopropylpyrazine. Regioselective lithiation (*n*-butyllithium, THF, ⁻78°C) of the chiral auxiliary produced the lithium enolate which afforded the adduct **2.47** from **2.46** with high diastereoselectivity (only one diastereomer was detectable in the ^{1}H NMR and ^{13}C

NMR spectra). Mild hydrolysis (2 N HCl) afforded the α-substituted amino acid methyl ester **2.48**. Amine **2.48** was protected as the NVOC carbamate, yielding **2.49** in 90% yield. The NVOC group was chosen as it can be removed by UV irradiation. *N*-Detosylation of **2.49** was performed using cesium carbonate in 2:1 THF–MeOH to yield **2.50** in 73% yield. Methyl ester **2.50** was then hydrolyzed to afford the free acid which was subsequently treated with chloroacetonitrile to afford the requisite cyanomethyl ester **2.51** in 54% overall yield. Treatment of cyanomethyl ester **2.51** with the tris(tetrabutylammonium) salt of pdCpA in anhydrous DMF afforded pdCpA ester **2.5** in 55% yield.

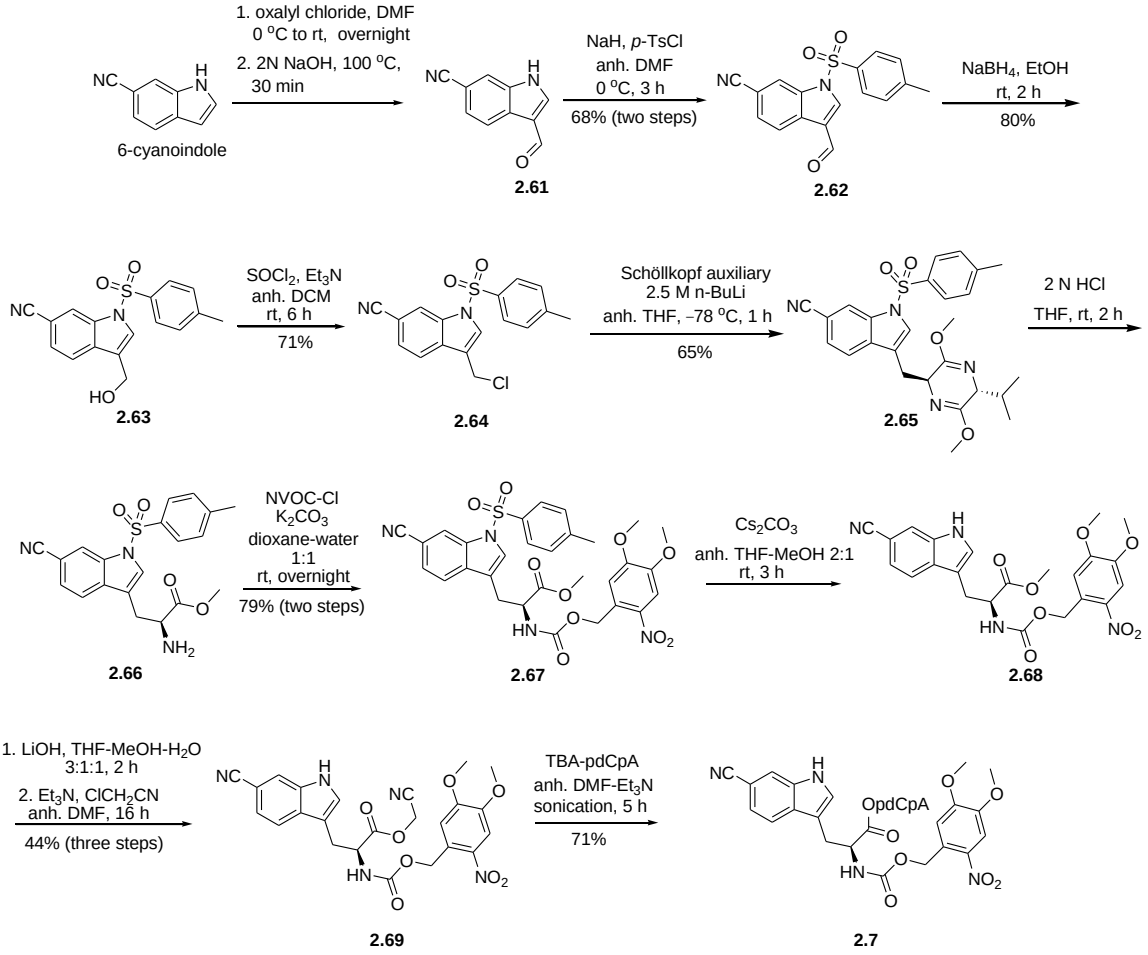
The synthesis of the aminoacylated pdCpA derivative of amino acid **Trp-6** (Scheme 2.7) was accomplished starting from commercially available pyrroloquinoline which was formylated to yield **2.52**. This compound was *N*-tosylated with *p*TsCl and NaH, affording **2.53** in 55% overall yield. Reduction of aldehyde **2.53** with $NaBH_4$ in EtOH then afforded alcohol **2.54** in 82% yield. Chlorination of **2.54** with thionyl chloride then provided **2.55**. Regioselective lithiation (*n*-butyllithium, THF, ⁻78°C) of the Schöllkopf reagent produced the lithium enolate which afforded the adduct **2.56** with high diastereoselectivity. Mild hydrolysis (2 N HCl) afforded the *α*-substituted amino acid methyl ester **2.57** which was protected as the NVOC carbamate to yield **2.58** in 92% yield. *N*-Detosylation of **2.58** using cesium carbonate in 2:1 THF–MeOH afforded ester **2.59**. Hydrolysis of **2.59** afforded the free acid, which was activated as cyanomethyl ester **2.60** in 40% overall yield. Treatment of cyanomethyl ester **2.60** with the tris(tetrabutylammonium) salt of pdCpA in anhydrous DMF gave the aminoacylated pdCpA containing amino acid **Trp-6** in 54% yield (Scheme 2.7).



Scheme 2.7. Synthesis of **Trp-6** and its Aminoacyl-pdCpA.

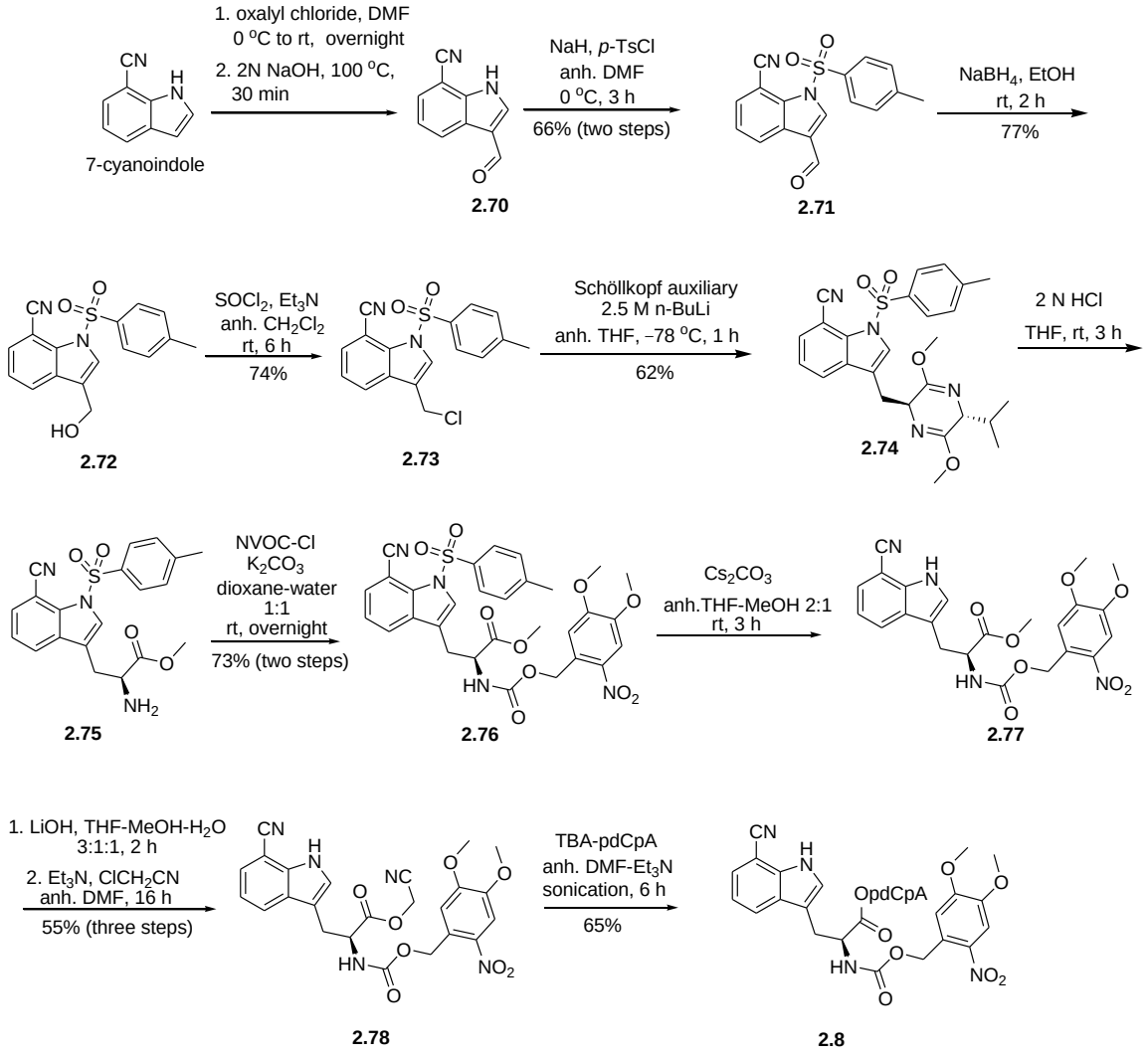
The synthesis of the aminoacylated pdCpA derivative of amino acid **Trp-7** (Scheme 2.8) was accomplished starting from commercially available 6-cyanoindole, which was formylated to yield **2.61**.[138] The latter was *N*-tosylated with *p*-TsCl and NaH, affording **2.62** in 68% overall yield. Reduction of the aldehyde moiety with $NaBH_4$ afforded alcohol **2.63** in 80% yield. Chlorination of **2.63** with thionyl chloride then provided **2.64** as a yellowish solid. Regioselective lithiation (*n*-butyllithium, THF, ⁻78°C) of the Schöllkopf reagent produced the lithium enolate which afforded adduct **2.65** with high diastereoselectivity. Mild hydrolysis (2 N HCl) provided *α*-substituted amino acid methyl ester **2.66** which was protected as the NVOC carbamate to yield **2.67** in 79%

overall yield. *N*-Detosylation of **2.67** using cesium carbonate in 2:1 THF–MeOH afforded ester **2.68**. Hydrolysis of **2.68** afforded the free acid, which was activated as cyanomethyl ester **2.69** in 44% overall yield (Scheme 2.8). Finally, treatment of cyanomethyl ester **2.69** with the tris(tetrabutylammonium) salt of pdCpA (TBA-pdCpA) in anhydrous DMF afforded pdCpA ester **2.7** in 71% yield.



Scheme 2.8. Synthesis of **Trp-7** and its Aminoacyl-pdCpA.

The synthesis of the aminoacylated pdCpA derivative of amino acid **Trp-8** employed a similar route (Scheme 2.9). Initially, commercially available 7-cyanoindole

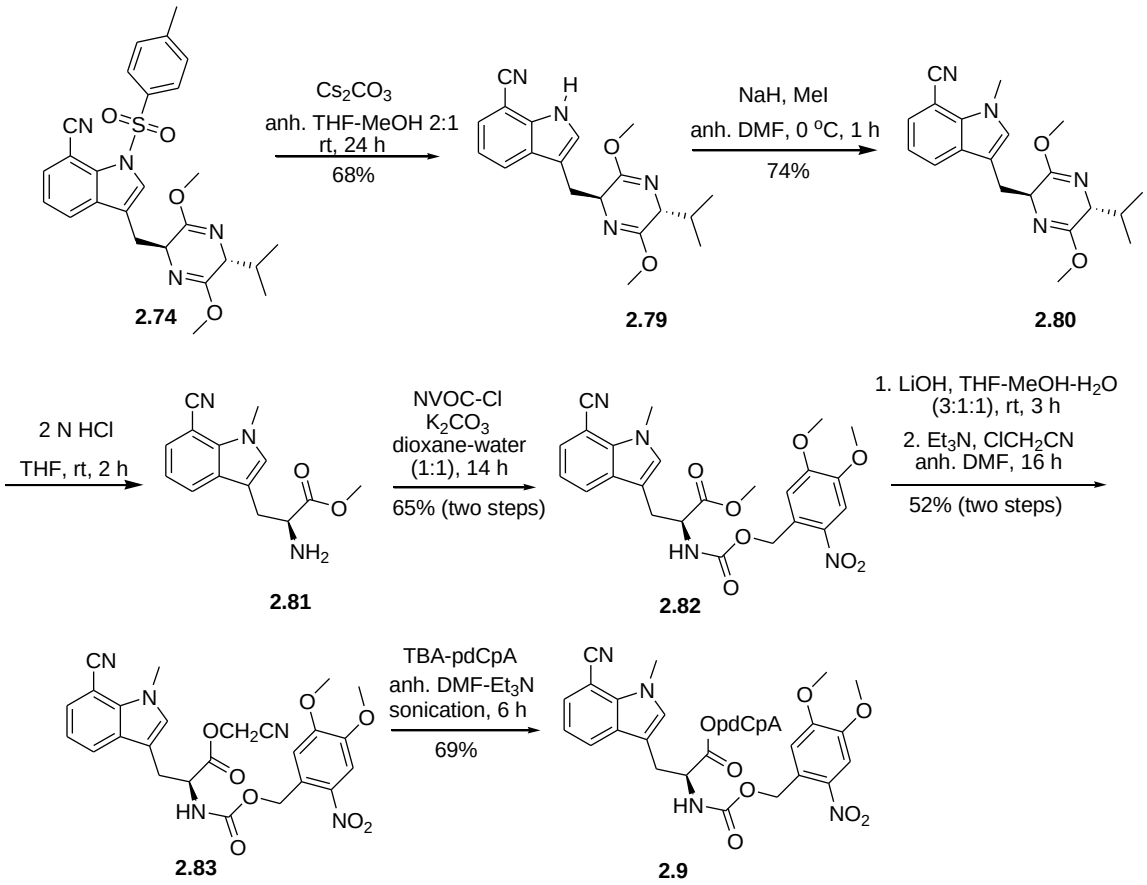


Scheme 2.9. Synthesis of **Trp-8** and its Aminoacyl-pdCpA.

was formylated to yield **2.70**, the latter of which was *N*-tosylated with *p*-TsCl and NaH, affording **2.71** in 66% overall yield. Treatment of aldehyde **2.71** with $NaBH_4$ afforded alcohol **2.72** in 77% yield. Chlorination of **2.72** with thionyl chloride then provided **2.73**. Regioselective lithiation of the Schöllkopf reagent produced the lithium enolate which afforded adduct **2.74** with high diastereoselectivity. Mild hydrolysis (2 N HCl) provided

α-substituted amino acid methyl ester **2.75** which was protected as the NVOC carbamate to yield **2.76** in 73% yield. *N*-Detosylation of **2.76** using cesium carbonate in 2:1 THF–MeOH afforded ester **2.77**. Hydrolysis of **2.77** afforded the free acid, which was activated as cyanomethyl ester **2.78** in 55% overall yield. Treatment of the cyanomethyl ester with a solution of the TBA-pdCpA in anhydrous DMF gave the corresponding aminoacylated pdCpA containing amino acid **Trp-8** in 65% yield (Scheme 2.9).

The synthesis of the aminoacylated pdCpA derivative of amino acid **Trp-9** was accomplished starting from the intermediate **2.74** (Scheme 2.10). *N*-Detosylation of **2.74** was performed using cesium carbonate in 2:1 THF–MeOH to yield **2.79** in 68% yield. *N*-methylation of **2.79** with methyl iodide (MeI) in presence of sodium hydride afforded **2.80** in 74% yield. Mild hydrolysis (2 N HCl) afforded the α-substituted amino acid methyl ester **2.81**. Amine **2.81** was protected as the NVOC carbamate to yield **2.82** in 65% overall yield. Methyl ester **2.82** was then hydrolyzed to afford the free acid which was subsequently treated with chloroacetonitrile to afford the requisite cyanomethyl ester **2.83** in 52% overall yield. Treatment of the cyanomethyl ester with a solution of the TBA-pdCpA in anhydrous DMF gave the corresponding aminoacylated pdCpA derivative **2.9** in 69% yield (Scheme 2.10).



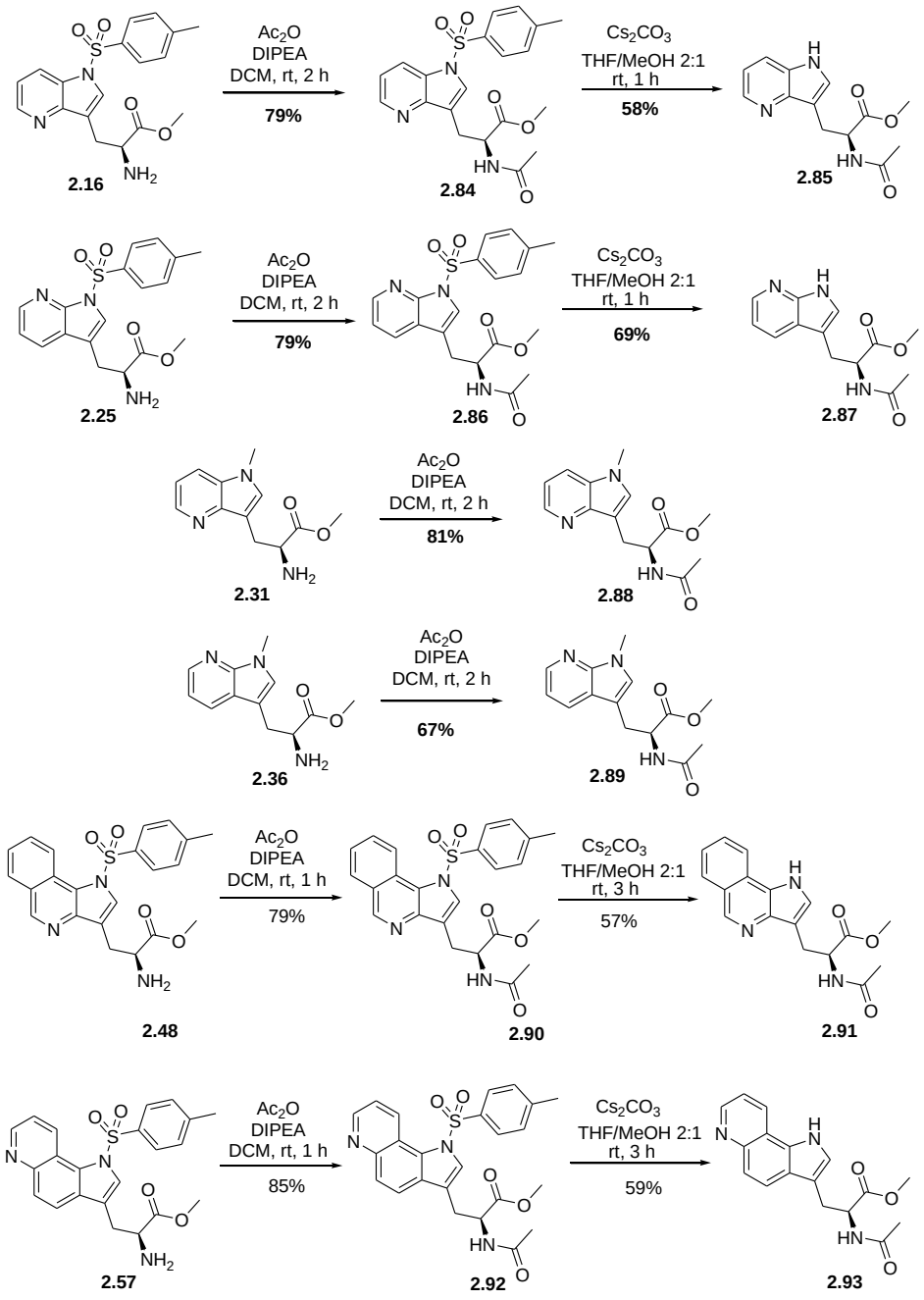
Scheme 2.10. Synthesis of **Trp-9** and its aminoacyl-pdCpA.

Synthesis of *N*-acetylated methyl esters of Trp analogues

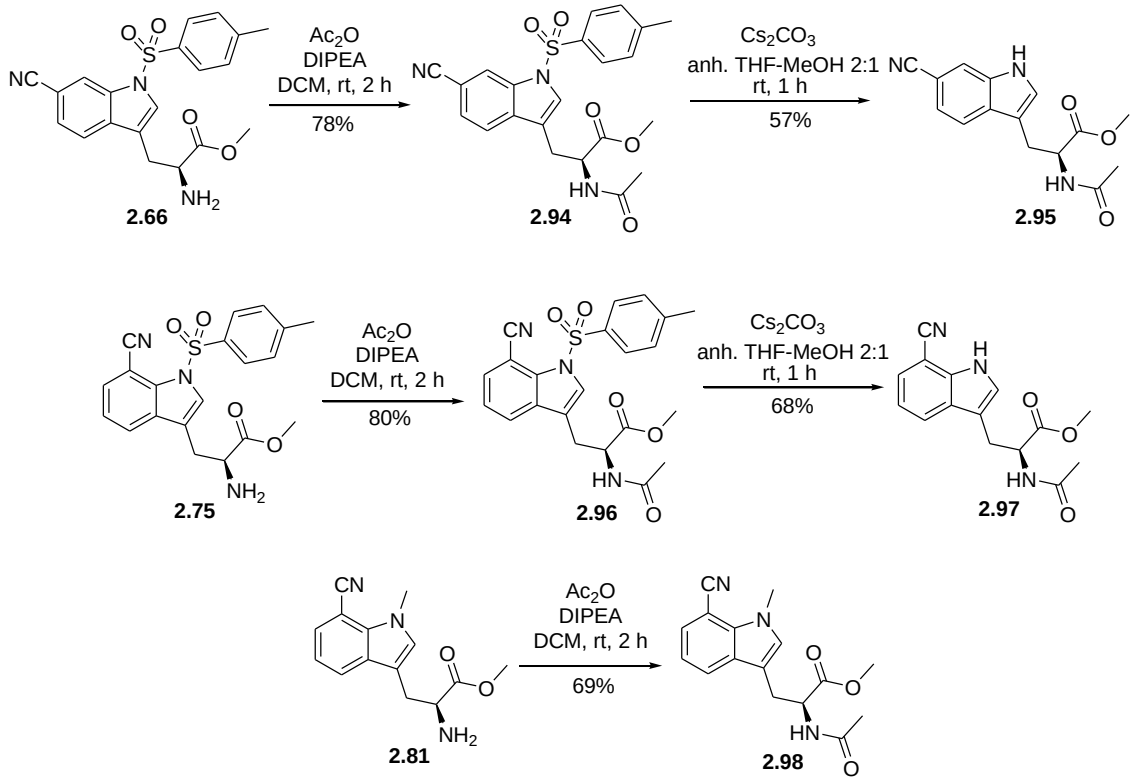
The synthesis of *N*-acetylated methyl esters of azaTrp analogues is illustrated in Scheme 2.11. Compounds **2.85**, **2.87**, **2.91** and **2.93** were prepared from intermediates **2.16**, **2.25**, **2.48** and **2.57**, respectively. They were acetylated using Ac_2O[139] and then detosylated using cesium carbonate. Compounds **2.88** and **2.89** were synthesized by acetylating intermediates **2.31** and **2.36** respectively.

The synthesis of the *N*-acetylated methyl esters of the cyanoTrp analogues is illustrated in Scheme 2.12. Compounds **2.95** and **2.97** were prepared from intermediates

2.66 and **2.75**; these were acetylated using Ac_2O and then detosylated using cesium carbonate. Compound **2.98** was synthesized by acetylating intermediate **2.81**.



Scheme 2.11. Synthesis of *N*-acetylated Methyl Esters of Azatryptophan Analogues.

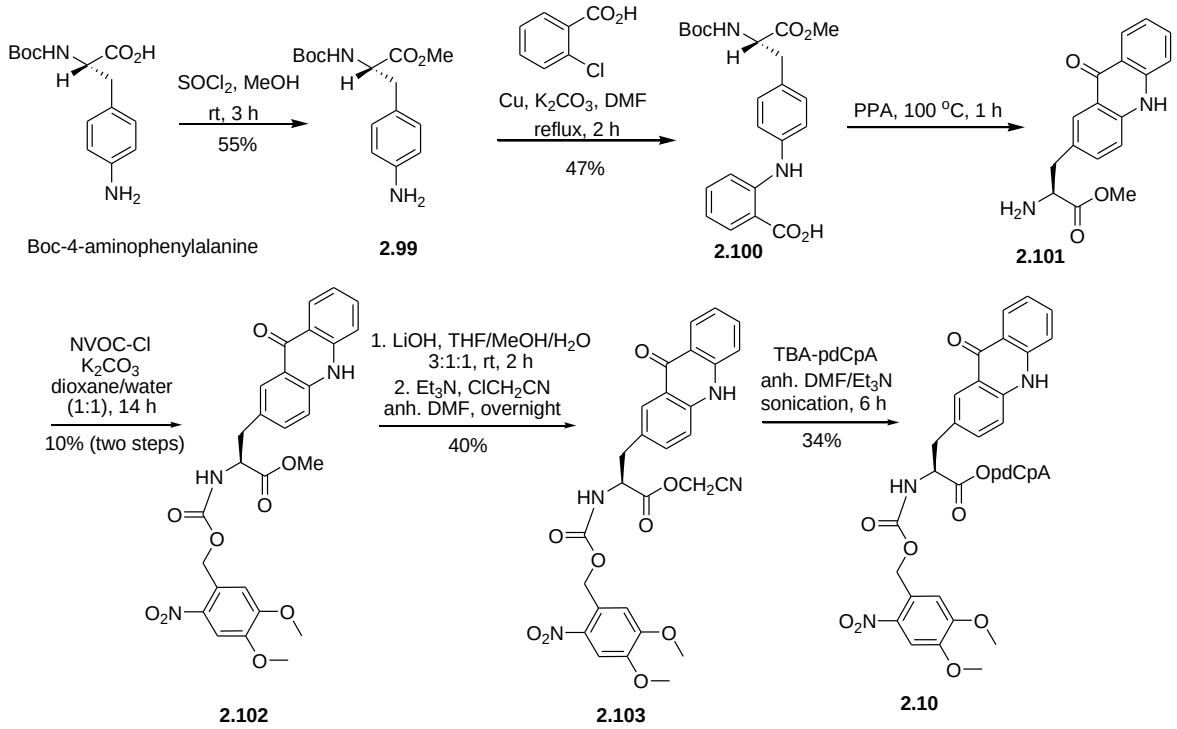


Scheme 2.12. Synthesis of *N*-acetylated Methyl Esters of Cyanotryptophan Analogues.

Synthesis of Acceptor Amino Acid Acd and its Aminoacyl-pdCpA Derivative

The synthesis of the aminoacylated pdCpA derivative of acceptor amino acid **Acd** was accomplished starting from commercially available Boc-4-aminophenylalanine which was first methylated with thionyl chloride in methanol to yield the methyl ester **2.99** (Scheme 2.13). Treatment of **2.99** with 2-chlorobenzoic acid then gave **2.100** in 47% yield.[128] Cyclodehydration of **2.100** to the acridinone derivative **2.101** with hot polyphosphoric acid (PPA) caused loss of the Boc protecting group.[128] Accordingly, free amine **2.101** was protected as the NVOC carbamate, yielding **2.102** in 10% overall yield from **2.100**. Methyl ester **2.102** was then treated with LiOH to afford the free acid, which was subsequently treated with chloroacetonitrile to afford the requisite cyanomethyl ester

2.103 in 40% yield. Treatment of the cyanomethyl ester with a solution of tris(tetrabutylammonium) salt of pdCpA in dry DMF gave the corresponding aminoacylated pdCpA **2.10** in 34% yield.



Scheme 2.13. Synthesis of **Acd** and its Aminoacyl-pdCpA.

Measurement of Photophysical Properties of Tryptophan Derivatives Trp-1–Trp-9

UV/vis absorption spectra (220-400 nm) were recorded using a Cary 60 UV/vis spectrophotometer. Fluorescence quantum yields (Table 2.1) of fluorescent compounds were determined using the gradient method.[140] Tryptophan derivatives **Trp-1**–**Trp-9** were dissolved in methanol, and solutions of each compound were made such that the UV absorptions at the maximum wavelength were 0.02, 0.04, 0.06, 0.08 and 0.1. Anthracene (Φ_F 0.27, λ_{ex} 340 nm), was used as a reference standard to calculate the fluorescence quantum yields of the tryptophan analogues according to the formula Φ_x =

$\Phi_s \times (Grad_x \times n_x^2)/(Grad_s \times n_s^2)$, where *Grad* is gradient of the plot of integrated intensity versus absorbance, *n* is the refractive index of the solvent, *s* is the standard of known Φ_F, and *x* is the tested sample.[140]

Table 2.1. Molar Absorptivities and Quantum Yields of *N*-Acetylated Methyl Esters of Trp Analogues in MeOH.

Trp analogue	$\lambda_{abs, max}$ (nm)	ε ($M^{-1}cm^{-1}$)	λ_{ex} (nm)	λ_{em} (nm)	Φ_F
Tryptophan	280	6900	280	340	0.18
Trp-1	290	7870	290	390	0.04
Trp-2	289	8920	289	371	0.02
Trp-3	292	7740	292	404	0.06
Trp-4	288	8070	288	391	0.30
Trp-5	262	18100	262	413	0.10
Trp-6	272	15500	272	421	0.03
Trp-7	290	10300	290	370	0.53
Trp-8	310	8100	310	390	0.40
Trp-9	315	8000	315	395	0.41

Activation of Suppressor $tRNA_{CUA}$ and Synthesis of Modified DHFRs

The individual *N*-NVOC protected aminoacylated pdCpA derivatives were ligated to a suppressor $tRNA_{CUA}$ lacking its 3′-terminal cytidine and adenosine residues ($tRNA_{CUA}$-C_{OH}) [12-14,141] via the agency of T4 RNA ligase. As shown in Figure 2.7, this assay afforded full length tRNA transcripts activated with amino acids **Trp-1** – **Trp-6**.

The NVOC protecting groups were then removed by exposure to high intensity UV light at 4 ^{o}C.[142] The aminoacyl-tRNAs so obtained were employed in an *in vitro* cell free transcription-translation system, which was programmed with DHFR DNA plasmids containing TAG codons at the positions corresponding to residues Trp22, Trp30 or Trp47 of DHFR.[143] As shown in Figure 2.8, each of the six azatryptophanyl-tRNAs afforded good suppression of the UAG codons at positions 22, 30 and 47 of the DHFR mRNAs, with suppression yields ranging from 12 to 65% compared to wild-type DHFR. The enzymatic activities of the modified DHFRs were judged by their ability to consume NADPH (Table 2.2). Since the substrates (dihydrofolate and NADPH) were present in excess of the enzyme, the rate constants (k_D) measured represent the enzyme turnover efficiency. Replacement of Trp22, which is in the Met20 loop subdomain of DHFR,[144] with tryptophan analogues **Trp-1** – **Trp-4** afforded DHFRs which consumed NADPH 22 – 51% as well as wild-type DHFR under the assay conditions. Substitution of tricyclic amino acids **Trp-5** and **Trp-6** at position 22 resulted in DHFRs which consumed NADPH 6 and 7% as well as wild type, respectively. After replacement of Trp30 and Trp47 by the same Trp analogues, the modified DHFRs had about the same NADPH consuming activity as wild type (Table 2.2).

Similarly, the individual *N*-NVOC protected aminoacylated pdCpA derivatives of **Trp-7** – **Trp-9**[145] were ligated to a suppressor $tRNA_{CUA}$ ($tRNA_{CUA}$-C_{OH}) by the use of T4 RNA ligase and the NVOC protecting groups were removed by exposure to high intensity UV light at 4 ^{o}C. The aminoacyl-tRNAs so obtained were employed in an *in vitro* cell free protein synthesizing system, which was programmed with DHFR DNA

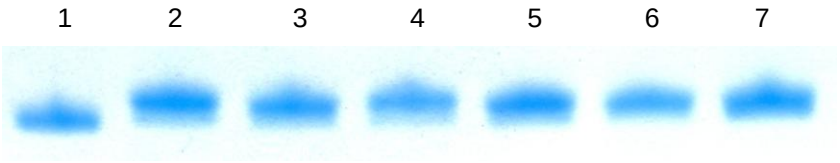


Figure 2.7. Ligation between the tRNA-C_{OH} (truncated $tRNA_{CUA}$) and aminoacylated pdCpA derivatives **2.1**-**2.6** as monitored by acidic polyacrylamide gel electrophoresis. Lane 1, truncated $tRNA_{CUA}$; lane 2, ligation of truncated $tRNA_{CUA}$ with **2.1**; lane 3, ligation of truncated $tRNA_{CUA}$ with **2.2**; lane 4, ligation of truncated $tRNA_{CUA}$ with **2.3**; lane 5, ligation of truncated $tRNA_{CUA}$ with **2.4**; lane 6, ligation of truncated $tRNA_{CUA}$ with **2.5**; lane 7, ligation of truncated $tRNA_{CUA}$ with **2.6**. The experiment was performed by Dr. Shengxi Chen.

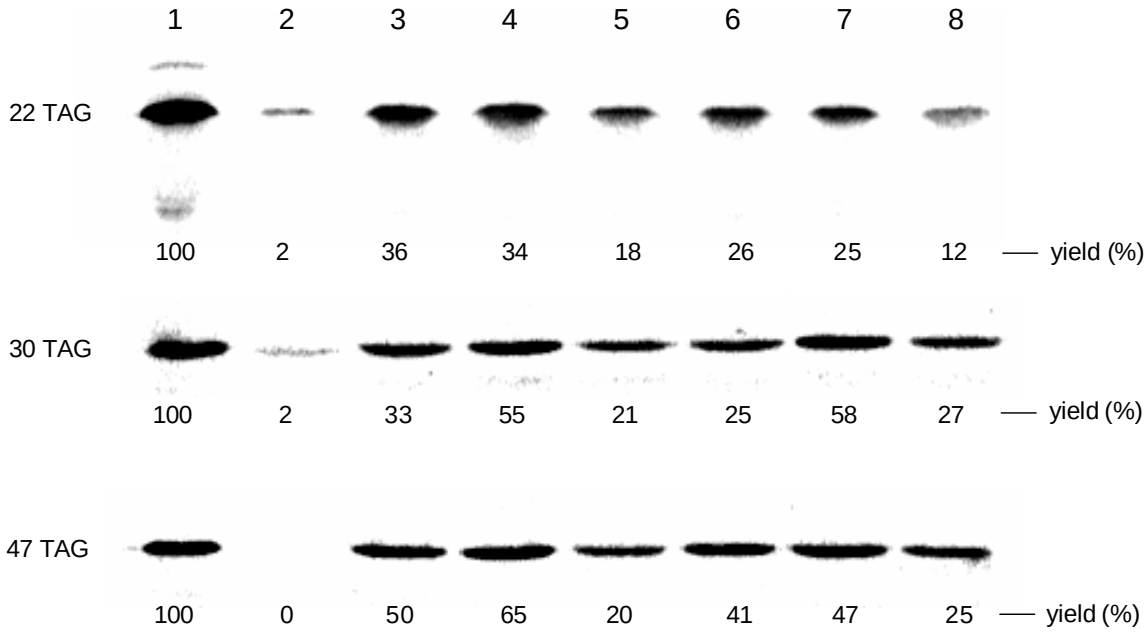


Figure 2.8. Autoradiogram of a 15% SDS-polyacrylamide gel (100 V, 2 h) illustrating the incorporation of tryptophan analogues into positions 22 (upper panel), 30 (middle panel) and 47 (lower panel) of DHFR. Lane 1, wild-type DHFR expression; lane 2, modified DHFR DNA in the presence of abbreviated suppressor $tRNA_{CUA}$-C_{OH}; lane 3, incorporation of amino acid **Trp-1**; lane 4, incorporation of amino acid **Trp-2**; lane 5, incorporation of amino acid **Trp-3**; lane 6, incorporation of amino acid **Trp-4**; lane 7, incorporation of amino acid **Trp-5**; lane 8, incorporation of amino acid **Trp-6**. Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. The experiment was performed by Dr. Shengxi Chen.

plasmids containing TAG codons at the positions corresponding to residues Trp22 or Trp74 of DHFR. Modified DHFR translation was carried out in an *in vitro* coupled transcription–translation system in the presence of the tryptophanyl-$tRNA_{CUA}$ derivatives.

Table 2.2. Enzymatic Activities of DHFRs Singly Modified at Positions 22, 30 or 47. The experiment was performed by Dr. Shengxi Chen.

	position 22 (Trp)	position 30 (Trp)	position 47 (Trp)
DHFR	k_D (s^{-1})	k_D (s^{-1})	k_D (s^{-1})
wild-type	12	12	12
Trp-1	6.1 ± 0.5[a]	13.2 ± 0.4	11.5 ± 0.4
Trp-2	5.4 ± 0.4	12.6 ± 0.4	12.4 ± 0.4
Trp-3	2.6 ± 0.2	12.0 ± 0.4	13.8 ± 0.4
Trp-4	3.7 ± 0.2	13.9 ± 0.5	13.2 ± 0.5
Trp-5	0.7 ± 0.1	12.5 ± 0.4	11.9 ± 0.4
Trp-6	0.8 ± 0.1	13.4 ± 0.5	12.8 ± 0.4

[a]Standard deviation is based on data from three experiments.

As shown in Table 2.3, each of the three cyanotryptophanyl-tRNAs afforded good suppression of the UAG codons at positions 22 and 74 of DHFR, with relative yields ranging from 15% to 41% compared to the cell free synthesis of wild-type DHFR. The enzymatic activities of the modified DHFRs were measured by their ability to consume NADPH (Table 2.3) under steady-state conditions. Replacement of Trp22, which is in the catalytically relevant Met20 loop subdomain of DHFR, with tryptophan analogues **Trp-7**

– **Trp-9** resulted in substantial reduction in enzyme activity (the turnover rate constants were found to be 9% to 14% of that obtained for wild-type DHFR[146] under the same assay conditions). We then replaced another tryptophan residue (Trp74), which is not located in the active site, with the same three tryptophan analogues. All of the modified DHFRs obtained had about the same NADPH consuming activity as wild type (Table 2.3).

Table 2.3. Expression Yields and Enzymatic Activities of DHFRs Singly Modified at Positions 22 and 74. The experiment was performed by Dr. Shengxi Chen.

	position 22 (Trp)		position 74 (Trp)	
DHFR	yield (%)	k_D (s^{-1})	yield (%)	k_D (s^{-1})
wild-type	100	12	100	12
Trp-7	15 ± 2	1.7 ± 0.1	37 ± 3	11.8 ± 0.5
Trp-8	15 ± 2	1.1 ± 0.1	35 ± 3	13.3 ± 0.5
Trp-9	19 ± 2	1.3 ± 0.1	41 ± 3	15.2 ± 0.6

[a] Standard deviation is based on data from three experiments.

Fluorescence and FRET in Modified DHFRs

We evaluated the fluorescence spectral properties of the tryptophan analogues when present in DHFR. The fluorescence emission spectra of the DHFRs containing the cyanotryptophans at position 74 are shown in Figure 2.9. The modified DHFRs were subjected to irradiation at 310 nm in order to minimize the fluorescence from Trp residues. The high quantum yield of **Trp-7** allowed us to observe strong fluorescence emission at 370 nm after excitation at 310 nm, even though this was 20 nm from its

maximum absorption wavelength. As expected, **Trp-8** and **Trp-9** exhibited substantially red-shifted emission spectra centered around 410 nm (Figure 2.9, green and blue traces respectively).

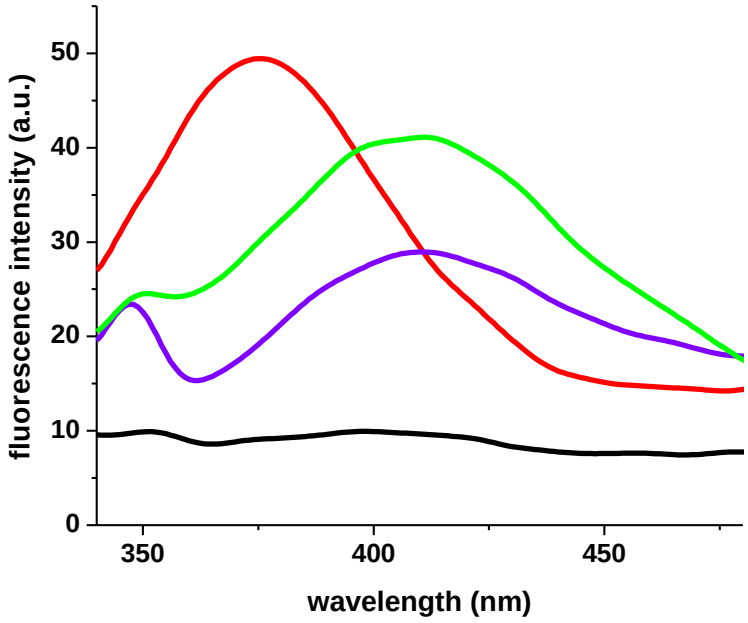
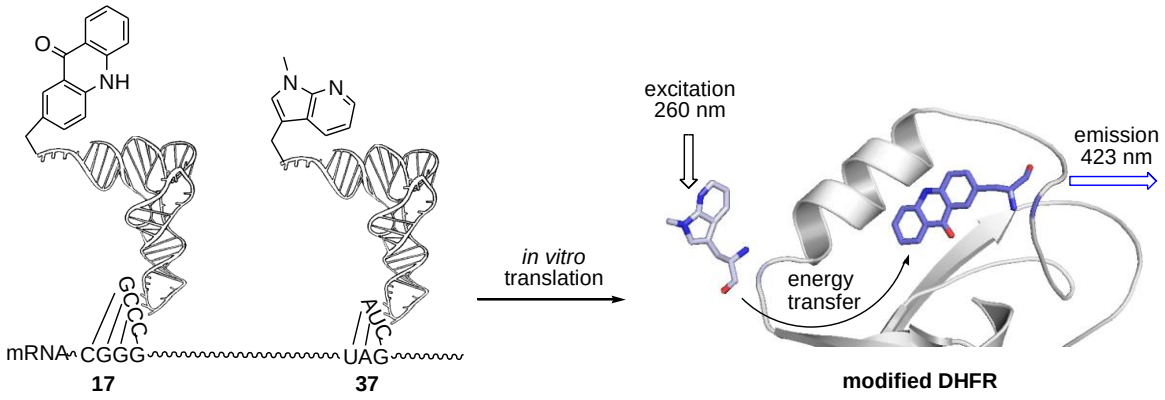


Figure 2.9. Fluorescence emission spectra of modified DHFRs containing amino acid **Trp-7** at position 74 (red trace), **Trp-8** at position 74 (green trace) and **Trp-9** at position 74 (blue trace) and wild-type DHFR (black).The spectra were recorded at pH 8.0 with λ_{ex} 310 nm. The experiment was performed by Dr. Shengxi Chen.

To explore the capability of the azatryptophan derivatives as FRET donors, we selected two analogues (**Trp-4** and **Trp-5**) and incorporated them into DHFR. As shown in Table 2.1, analogue **Trp-4** has a higher molar absorbance (8070) and a higher quantum yield (0.30) than tryptophan (6900 and 0.18, respectively); and analogue **Trp-5** has a 2.6-fold greater molar absorbance than tryptophan, albeit a lower (0.10) quantum yield. Both of the analogues exhibit stronger fluorescence emission and should thus minimize the background from the tryptophans in DHFR. The emission wavelength maxima of tryptophan analogues **Trp-4** and **Trp-5** are at 391 nm and 413 nm, respectively (Table

2.1). To utilize the emission of these two fluorescence donors in FRET, we selected acridon-2-ylalanine (**Acd**) as the fluorescence acceptor; this fluorophore has absorption peaks at 388 and 407 nm in H_2O and emission peaks at ~ 420 and 450 nm. Once the FRET pairs were selected, we calculated the Förster distance (R_0) of these donors and acceptor, following the standard protocol.[129] The R_0 between **Trp-4** and **Acd** is 27.4 Å; and the R_0 between **Trp-5** and **Acd** is 29.5 Å. We replaced Glu17 with the acceptor **Acd** to study the conformational changes in DHFR structure via FRET. For the donor position, we selected Asn37 to fit the calculated R_0 value, as its distance to E17 is 33 Å (PBD entry 1RA1). As shown in Scheme 2.14, by decoding nonsense codon UAG and four-base codon CGGG in the presence of an aminoacyl-$tRNA_{CUA}$ and aminoacyl-$tRNA_{CCCG}$, the donors (**Trp-4** or **Trp-5**) and the acceptor (**Acd**) amino acids were incorporated into DHFR at positions 37 and 17, respectively. The incorporation yields were 8.3 and 8.8%, relative to wild type (Figure 2.10). As shown in Table 2.4, even though the acceptor **Acd** at position 17 decreased the activity of DHFR to 59% of



Scheme 2.14. Strategy Employed for Incorporation of a Pair of Fluorophores into DHFR at Positions 17 and 37 for a FRET Study.

wild type, the donors (**Trp-4** or **Trp-5**) did not further perturb the activity of DHFR when introduced into position 37. The doubly modified DHFRs were excited at 260 nm, which was chosen in order to decrease the interference of the remaining tryptophans in DHFR (having λ_{max} 280 nm). As shown (Figure 2.11), the modified DHFR containing the fluorescent acceptor **Acd** at position 17 and the donor **Trp-5** at position 37 exhibited a FRET emission peak at 423 nm. Similarly, the modified DHFR containing the fluorescent acceptor **Acd** at position 17 and the donor **Trp-4** at position 37 exhibited a stronger FRET emission at 423 nm. Comparatively, the four remaining tryptophans in DHFR displayed only very weak FRET emissions at the same wavelength (Figure 2.11, blue trace).

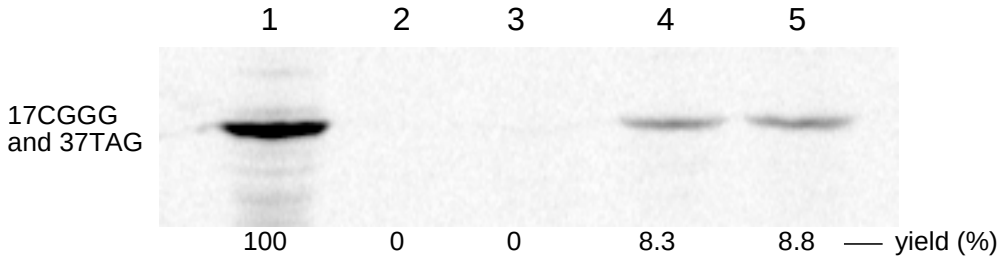


Figure 2.10. Autoradiogram of a 15% SDS-polyacrylamide gel (100 V, 2 h) illustrating the incorporation of **Acd** and tryptophan analogues into positions 17 and 37 of DHFR. Lane 1, wild-type DHFR expression; lane 2, modified DHFR DNA (17CGGG; 37TAG) in the presence of abbreviated suppressor $tRNA_{CCCG}$-C_{OH}; lane 3, modified DHFR DNA in the presence of L-acridon-2-ylalanyl-$tRNA_{CCCG}$; lane 4, incorporation of amino acids **Trp-4** and **Acd**; lane 5, incorporation of amino acids **Trp-5** and **Acd**. The experiment was performed by Dr. Shengxi Chen.

To explore the capability of cyanotryptophan derivatives as FRET donors, we selected 6-CNTrp (**Trp-7**). As shown in Table 2.1, the emission wavelength (λ_{em}) of fluorescence donor **Trp-7** was at 370 nm. As the fluorescence acceptor we selected L-(7-hydroxycoumarin-4-yl) ethylglycine (**HCO**)[48] whose excitation and emission maxima

were found to be at 345 and 440 nm, respectively. For this FRET pair, we calculated the Förster distance (R_0) as 28.3 Å by following the standard protocol. As noted (Table 2.3), position 74 tolerated the introduction of all the cyanotryptophans without significant loss

Table 2.4. Enzymatic Activities of Modified DHFRs Containing FRET Pairs.

DHFR	k_D (s^{-1})
wild-type	12
17**Acd**37**Trp-4**	7.1 ± 0.4
17**Acd**37**Trp-5**	6.1 ± 0.4
17**Acd**	7.1 ± 0.5

[a]Standard deviation based on data from three experiments.

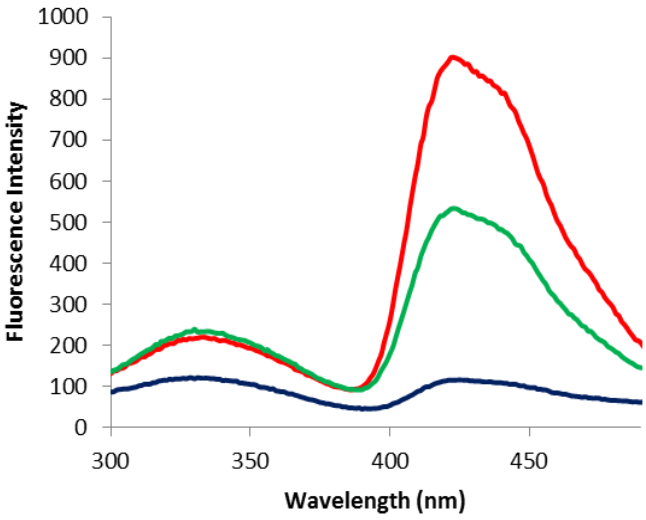


Figure 2.11. Fluorescence emission spectra of DHFRs containing amino acid **Acd** at position 17 (blue trace), **Acd** at position 17 and **Trp-4** at position 37 (red trace), and **Acd** at position 17 and **Trp-5** at position 37 (green trace). The spectra were recorded at pH 8.0 following excitation at 260 nm. The experiment was performed by Dr. Shengxi Chen.

of enzyme function, it was chosen as the position for the donor; for the acceptor position, we chose position 17. Its distance to W74 is 25 Å (PBD 1RA1), which is suitable for studying FRET between this pair. Using the same strategy applied for azatryptophans, the acceptor (**HCO**) and donor (**Trp-7**) amino acids were incorporated into a single protein of DHFR at positions 17 and 74, respectively. The incorporation yield was 13% relative to wild type and the rate of NADPH consumption decreased to 9.4 ± 0.5 s^{-1} compared to 12 s^{-1} for wild type. Then the doubly-modified DHFR was excited at 310 nm in order to decrease the interference from tryptophan (λ_{max} 280 nm). As shown in Figure 2.12, the modified DHFR containing the fluorescence acceptor **HCO** at position 17 and donor **Trp-7** at position 74 exhibited a substantial FRET signal at 460 nm.

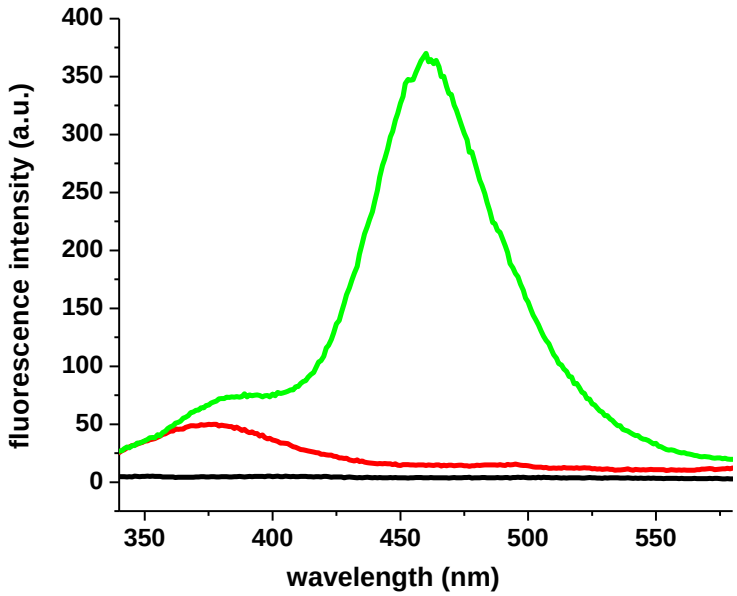


Figure 2.12. Fluorescence emission spectra of DHFRs containing amino acid **Trp-7** at position 74 (red trace), **HCO** at position 17 and **Trp-7** at position 74 (green trace), and wild-type DHFR (black). The spectra were recorded at pH 8.0 with λ_{ex} 310 nm. The Experiment was performed by Dr. Shengxi Chen.

4.3. Discussion

As part of ongoing efforts to develop smaller and sensitive fluorophores that can be readily accommodated within a protein without perturbation of conformation and function, we synthesized a series of azatryptophans and cyanotryptophans. Recently, *N*-methylated 4- and 7-azaindoles have been found to have fluorescence properties better than the parent 4- and 7-azaindoles because they retain the pronounced red shift characteristic of the parent 4- and 7-azaindoles, but with more intense fluorescence.[120] Therefore, along with 4- and 7-azatryptophans, we also synthesized *N*-methylated 4- and 7-azatryptophans as well as two tricyclic tryptophan derivatives (Figure 2.3). Likewise, along with 6-cyanotryptophan and 7-cyanotryptophan, *N*-methylated 7-cyanotryptophan was synthesized (Figure 2.3). Being more hydrophobic, the *N*-methylated analogues should also stabilize the hydrophobic core of proteins.

In order to synthesize the respective aminoacylated tRNAs, the pdCpA derivatives of tryptophan analogues were prepared from their respective amino acids. Enzymatic synthesis and resolution is often used for the synthesis of naturally occurring chiral α–amino acids. However, this strategy is frequently not useful for synthesizing amino acids with non-natural side chains. To realize the asymmetric synthesis of the Trp analogues, a stereoselective strategy utilizing the Schöllkopf chiral reagent has been adopted and all the syntheses started from commercially available indoles except **Trp-5**. For the asymmetric synthesis of **Trp-5**, the requisite indole **2.42** was prepared in four steps from commercially available 4-bromoisoquinoline according to the method of Dupas et al.[135] with some modifications. The synthesis began with amination of 4-bromoisoquinoline (Scheme 2.5). The reaction was carried out using Cu/CuCl couple,

which induced the transformation at much lower temperature than the reported procedure (autoclave, aqueous ammonia, $CuSO_4$, 150°–200°C).[147] Iodination with iodine/silver sulfate provided 4-amino-3-iodoisoquinoline (**2.40**) in satisfactory yield. Sonogashira coupling of trimethylsilylacetylene (TMSA) and 4-amino-3-iodoisoquinoline (**2.40**) proceeded very smoothly at room temperature to afford the desired ethynyl derivative **2.41**, whereas the literature procedure involved coupling with 4-amino-3-bromoisoquinoline at higher temperature in lower yield. The aminoethynyl derivative **2.41** was then heated to reflux with $NaNH_2$ in DMF to effect cyclization into key intermediate **2.42**. The formylation reaction of azaindoles were performed using Duff reaction conditions,[131] whereas the cyanoindoles were formylated by Vilsmeier–Haack reaction[138]. Tosyl protecting group was chosen for indole NH protection because it was expected to survive untill the last step. Finally it was removed using cesium carbonate in 2:1 THF–MeOH. The asymmetric synthesis of the amino acid precursor was carried out using the Schöllkopf reagent [(*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine] which is basically a valine derived bis-lactim ether. The auxiliary could be deprotonated at the prochiral α-carbon, and the resulting enolate attacked the chloride electrophiles to yield adducts with high diastereoselectivity (only one diastereomer was detectable in the ^1H NMR and ^{13}C NMR spectra). The enolate is essentially planar, and the steric bulk of the isopropyl group directed the incoming electrophile to attack from the opposite face, yielding *trans* adducts. Hydrolysis, typically under mild acidic conditions, yielded the α-substituted amino acids with high enantiopurity and the free amines were protected as NVOC carbamates. The NVOC protecting group was chosen as it can be readily deprotected by treatment with UV irradiation at the tRNA level without any undesirable

side reaction. Protected methyl esters were hydrolyzed to form NVOC protected acids, the latter of which were activated as the corresponding cyanomethyl esters by treatment with chloroacetonitrile in dry DMF in the presence of triethylamine. The key cyanomethyl esters of the *N*-protected amino acids were coupled with pdCpA TBA salt to give dinucleotide esters (**2.1**–**2.9**) which were obtained as colorless solids after HPLC purification and lyophilization. Misacylated tryptophanyl tRNAs were produced by ligating abbreviated tRNA, lacking the terminal cytidine and adenosine moieties at the 3′-end, with the pdCpA derivatives **2.1**–**2.9** using T4 tRNA ligase. The ligase is an ATP dependent enzyme that catalyzes 3′→5′ phosphodiester bond formation between single-stranded RNAs. The activated tRNAs were then deprotected by UV irradiation to afford the free aminoacyl tRNAs. The activated suppressor tRNA was employed in an *in vitro* protein synthesizing system programmed with DHFR mRNA having a UAG codon at predetermined positions of the elaborated protein to introduce Trp analogues into specific positions of DHFR.

According to the X-ray crystallographic structure of DHFR, residue Trp22 is involved in substrate binding through H-bonds between the residue and an ordered water molecule.[148] Replacement of this residue may disorder substrate binding and affect the function of DHFR. This is consistent with the finding that incorporation of Trp analogues into position 22 reduced the activity of the modified DHFRs significantly compared to wild type. Replacement of the tryptophan residues Trp30, Trp47 or Trp74, which are not located in the active site, with the tryptophan analogues afforded modified DHFRs having the same NADPH consuming activity as wild type. The results demonstrated that these tryptophan derivatives have properties as fluorescence donors suitable for minimal

perturbation of protein structures, thus potentially allowing the study of conformational changes in DHFR.

Acridon-2-ylalanine (**Acd**) or L-(7-hydroxycoumarin-4-yl) ethylglycine (**HCO**) has been used as a fluorescence acceptor for FRET studies. **Acd** has a small size (222 $Å^3$), long lifetime ($\tau \sim 15$ ns) and high quantum yield (Φ_F 0.95 in water). **HCO** also has a small size, relatively large Stokes shift, high quantum yield (Φ_F 0.63 in water) and a blue wavelength fluorescence emission. The Glu17 residue of *E. coli* DHFR is located in the Met 20 loop, which exhibits conformational changes upon substrate and cofactor binding and product release.[148] Position 17 of DHFR tolerates small fluorescent amino acids.[47,48] Therefore, we replaced Glu17 with the acceptor **Acd**/**HCO** to study the conformational changes in DHFR structure via FRET.

Several tryptophan analogues have been prepared and characterized with respect to their photophysical properties, and incorporated into different positions of dihydrofolate reductase. Three of the tryptophan analogues were found to support FRET efficiently. The results demonstrate the utility of these tryptophan analogues as participants in energy transfer experiments in DHFR, even in the presence of other unmodified tryptophans.

2.4. Experimental Procedures

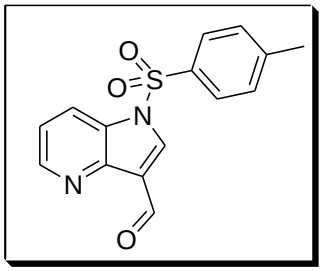
Materials. The chemicals used were purchased from Aldrich Chemical Co., Sigma Chemical Co. or Combi-Blocks and were used without further purification. Anhydrous methanol and DMF were used as purchased. Tetrahydrofuran and dichloromethane were distilled from sodium/benzophenone and calcium hydride,

respectively. The tris(tetrabutylammonium) salt of pdCpA was prepared by passing pdCpA through the activated TBA form of Dowex 50W×8 (200-400 mesh).

General Experimental Procedures. All experiments requiring anhydrous conditions were conducted in flame-dried glassware fitted with rubber septa under a positive pressure of dry nitrogen, unless otherwise noted. Reactions performed at room temperature unless indicated otherwise. Analytical thin layer chromatography was performed using glass plates pre-coated with silica gel (0.25 mm, 60 Å pore size, 230-400 mesh, Silicycle) impregnated with a fluorescent indicator (254 nm). TLC plates were visualized by exposure to ultraviolet light (UV). Flash column chromatography was performed employing silica gel (60 Å pore size, 40-63 μm, standard grade, Silicycle). An acetone bath was cooled to the appropriate temperature by addition of small portions of dry ice.

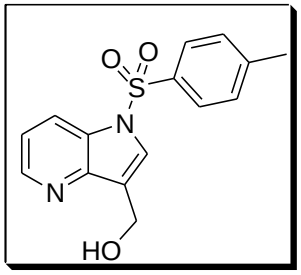
Instrumentation. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian INOVA 400 (400 MHz) and Varian INOVA 500 (500 MHz) spectrometers at 25 °C. Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to residual protium in the NMR solvent ($CDCl_3$, DMSO-d_6 and CD_3OD). Splitting patterns are designated as follows: s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectrometric data were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or the Michigan State Mass Spectrometry Facility. HPLC purification was performed with a Waters 600 pump coupled with a Varian ProStar 340 detector and a Grace Econosil C_{18} column (250 × 10 mm, 5 μm). The tetra-*n*-

butylammonium (TBA) salt of pdCpA was prepared using Dowex 50WX8, 200–400 mesh activated in its TBA form.

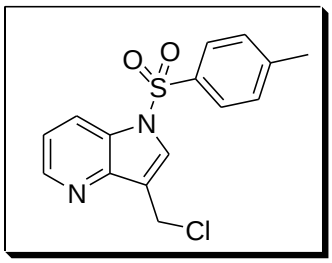


1-Tosyl-1*H*-pyrrolo[3,2-*b*]pyridine-3-carbaldehyde (2.12). To a stirred solution containing 1.50 g (12.7 mmol) of 1*H*-pyrrolo[3,2-*b*]pyridine in 20 mL of 1:1 AcOH–H_2O was added 2.67 g (19.0 mmol) of hexamethylenetetramine (HMTA). The reaction mixture was heated at reflux under argon overnight. The reaction mixture was cooled to 0 °C and the formed precipitate was filtered and dried under vacuum. 1*H*-pyrrolo[3,2-*b*]pyridine-3-carbaldehyde (**2.11**) was obtained as a light yellow solid and was used directly in the next step without further purification. To a stirred solution containing 487 mg (3.33 mmol) of 1*H*-pyrrolo[3,2-*b*]pyridine-3-carbaldehyde (**2.11**) in 20 mL of anhydrous DMF at 0 °C was added 119 mg (4.99 mmol) of NaH. The reaction mixture was stirred at 0 °C for 10 min under argon and then 954 mg (4.99 mmol) of *p*-TsCl was added. The reaction mixture was stirred at 0 °C under argon for 3 h, diluted with 100 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 4:1 hexanes–ethyl acetate gave 1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridine-3-carbaldehyde (**2.12**) as an off-white solid: yield 691 mg (23% for two steps); silica gel TLC R_f 0.35 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.31 (s, 3H), 7.23-7.29 (m, 3H),

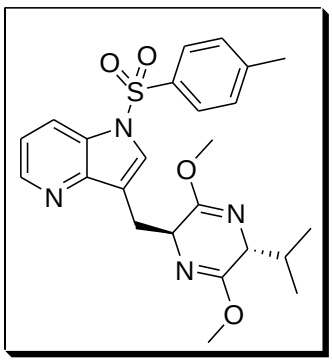
7.77 (d, 2H, *J* = 8.0 Hz), 8.21 (d, 1H, *J* = 8.0 Hz), 8.38 (s, 1H), 8.62 (d, 1H, *J* = 4.0 Hz) and 10.29 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 120.4, 121.2, 121.4 127.3, 128.8, 130.6, 134.1, 134.8, 144.9, 146.8, 147.8 and 184.9; mass spectrum (APCI), *m*/*z* 301.0639 $(M+H)^+$ ($C_{15}H_{13}N_2O_3S$ requires *m*/*z* 301.0647).



1-Tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)methanol (2.13). To a suspension of 880 mg (2.93 mmol) of 1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridine-3-carbaldehyde (**2.12**) in 15 mL of EtOH was added 222 mg (5.86 mmol) of $NaBH_4$ and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with 100 mL of satd aq $NaHCO_3$ and the formed precipitate was filtered and dried under vacuum. 1-Tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)methanol (**2.13**) was obtained as an off-white solid: yield 770 mg (87%); silica gel TLC R_f 0.1 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.34 (s, 3H), 4.92 (s, 2H), 7.22-7.26 (m, 3H), 7.73-7.76 (m, 3H), 8.25 (d, 1H, *J* = 8.0 Hz) and 8.46 (d, 1H, *J* = 4.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.8, 56.8, 119.5, 121.3, 122.9, 126.2, 127.0, 129.1, 130.3, 135.1, 145.7, 145.9 and 147.6; mass spectrum (APCI), *m*/*z* 303.0799 $(M+H)^+$ ($C_{15}H_{15}N_2O_3S$ requires *m*/*z* 303.0803).

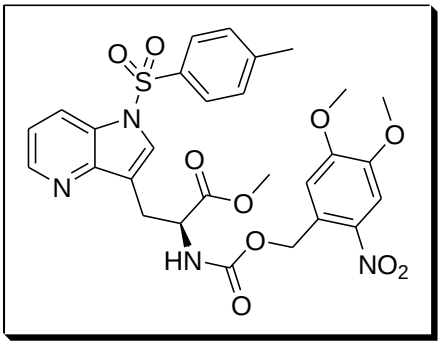


3-(Chloromethyl)-1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridine (2.14). To a cooled (⁻10 °C) solution containing 770 mg (2.55 mmol) of 1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)methanol (**2.13**) in 15 mL of anhydrous CH_2Cl_2 and 1.42 mL (1.03 g, 10.2 mmol) of Et_3N was added dropwise 0.37 mL (606 mg, 5.09 mmol) of $SOCl_2$. The reaction mixture was left to warm slowly to room temperature and stirred for 6 h, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 4:1 hexanes–ethyl acetate gave 3-(chloromethyl)-1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridine (**2.14**) as a yellow oil: yield 579 mg (73%); silica gel TLC R_f 0.45 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.19 (s, 3H), 4.77 (s, 2H), 7.11-7.20 (m, 3H), 7.69-7.72 (d, 2H, J = 8.0 Hz), 7.83(s, 1H), 8.18 (dd, 1H, J = 8.4 and 1.2 Hz) and 8.48 (dd, 1H, J = 4.8 and 1.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.3, 35.4, 119.5, 119.8, 120.8, 126.6, 127.9, 128.4, 129.9, 134.4, 145.6, 145.9 and 146.1; mass spectrum (APCI), *m*/*z* 321.0474 $(M+H)^+$ ($C_{15}H_{14}N_2O_2SCl$ requires *m*/*z* 321.0465).



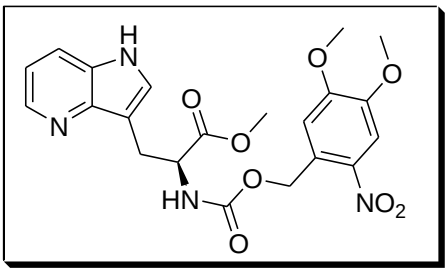
3-(((2*S*,5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridine (2.15). To a stirred solution containing 0.34 mL (363 mg, 1.97 mmol) of Schöllkopf's reagent in 5 mL of anhydrous THF at ⁻78 °C was added 0.25 mL

(172 mg, 2.68 mmol) of 2.5 M BuLi. The reaction mixture was stirred at −78 °C for 30 min under argon and then a solution containing 575 mg (1.79 mmol) of 3-(chloromethyl)-1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridine (**2.14**) in 5 mL of anhydrous THF was added. The reaction mixture was stirred at −78 °C for 1 h under argon, then diluted with 50 mL of satd aq NH_4Cl and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 2:1 hexanes–ethyl acetate gave the expected product 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridine (**2.15**) as a yellow oil: yield 513 mg (62%); silica gel TLC R_f 0.35 (4:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.55 (d, 3H, J = 8.0 Hz), 0.85 (d, 3H, J = 8.0 Hz), 1.96-2.06 (m, 1H), 2.22 (s, 3H), 3.10-3.12 (m, 1H), 3.30-3.32 (m, 2H), 3.54 (s, 3H), 3.56 (s, 3H), 3.61-3.65 (m, 1H), 4.27-4.29 (m, 1H), 7.06-7.11 (m, 3H), 7.50 (s, 1H), 7.60 (d, 1H, J = 8.0 Hz), 8.12 (dd, 1H, J = 8.4 and 1.2 Hz) and 8.41 (dd, 1H, J = 4.8 and 1.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.5, 18.9, 21.4, 28.2, 31.5, 52.1, 52.2, 55.1, 60.4, 118.8, 119.9, 120.5, 126.5, 127.3, 128.2, 129.9, 134.9, 145.1, 145.6, 148.5, 162.7 and 163.7; mass spectrum (APCI), *m*/*z* 469.1913 $(M+H)^+$ ($C_{24}H_{29}N_4O_4S$ requires *m*/*z* 469.1909).



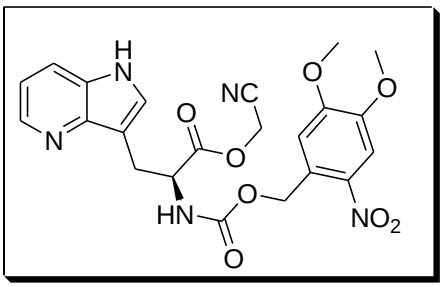
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (2.17). To a stirred solution containing 513 mg (1.09 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridine (**2.15**) in 16 mL of THF at 0 °C was added 14 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then slowly poured into 50 mL of satd aq $NaHCO_3$ and then extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. Methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.16**) was obtained as a yellow oil and was used directly in the next step without further purification. To a stirred solution containing 165 mg (0.44 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl) propionate (**2.16**) in 2 mL of dioxane-water (1:1) was added 214 mg (1.55 mmol) of K_2CO_3 followed by 123 mg (0.57 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 14 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.17**) as a yellow oil: yield 216 mg (33% for two steps); silica gel TLC R_f 0.50 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.28 (s, 3H), 3.26-3.28 (m, 2H), 3.53 (s, 3H), 3.72 (s, 3H), 3.86 (s, 3H), 4.60-4.62 (m, 1H), 5.36 (d, 1H, J = 16 Hz), 5.55 (d, 1H, J = 16 Hz), 6.93 (s, 1H), 7.18-7.23 (m, 3H), 7.61-7.68 (m, 3H), 7.78 (d, 1H, J = 7.2 Hz), 8.20 (d, 1H, J =8.0 Hz) and 8.47 (d, 1H, J = 4.4 Hz); ^{13}C

NMR ($CDCl_3$, 100 MHz) δ 21.5, 26.8, 52.1, 54.6, 56.1, 56.3, 63.3, 107.9, 109.1, 118.4, 119.5, 121.2, 126.6, 127.9, 128.7, 128.9, 130.1, 134.6, 139.1, 145.5, 145.7, 147.6, 147.7, 153.7, 155.8 and 171.4; mass spectrum (APCI), *m*/*z* 613.1609 $(M+H)^+$ ($C_{28}H_{29}N_4O_{10}S$ requires *m*/*z* 613.1604).



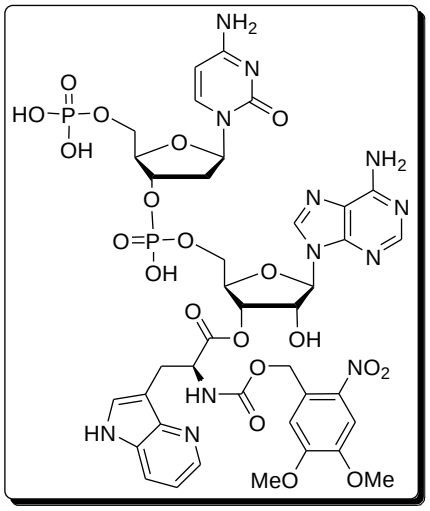
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (2.18). To a stirred solution containing 151 mg (0.25 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.17**) in 3 mL of 2:1 THF–methanol was added 261 mg (0.74 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 2 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.18**) as a yellow oil: yield 42.0 mg (38%); silica gel TLC R_f 0.30 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.36-3.38 (m, 2H), 3.60 (s, 3H), 3.76 (s, 3H), 3.90 (s, 3H), 4.59-4.64 (m, 1H), 5.43 (d, 1H, *J* = 16 Hz), 5.65 (d, 1H, *J* = 16 Hz), 7.03 (s, 1H), 7.08-7.12 (m, 1H), 7.23 (d, 1H, *J* = 2 Hz), 7.61-7.66 (m, 2H), 8.43 (d, 1H, *J* =4.4 Hz) and 8.63 (d, 1H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.1, 52.1, 55.9, 56.1, 56.3, 63.4,

107.8, 108.9, 110.9, 117.0, 119.1, 127.2 129.3, 129.4, 138.9, 142.2, 144.9, 147.6, 153.8, 156.4 and 172.2; mass spectrum (APCI), *m*/*z* 459.1507 $(M+H)^+$ ($C_{21}H_{23}N_4O_8$ requires *m*/*z* 459.1516).

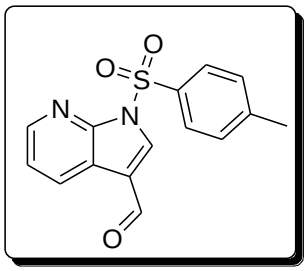


Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (2.19). To a stirred solution containing 42.0 mg (0.09 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*b*]pyridin-3-yl) propionate (**2.18**) in 1 mL of 1:3:1 water–THF–methanol was added 275 μL (6.46 mg, 0.27 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 3 h, and then concentrated under diminished pressure. The residue was redissolved in 1 mL of anhydrous DMF under argon. To the stirred solution was added 38.0 μL (28.0 mg, 0.27 mmol) of Et_3N followed by 17.0 μL (20.0 mg, 0.27 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h, and then diluted with 20 mL of EtOAc. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave the expected product cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.19**) as a light yellow solid: yield 23.1 mg (52%); silica gel TLC R_f 0.5 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.45 (d, 2H, *J* = 4.4 Hz), 3.75 (s, 3H), 3.92 (s, 3H), 4.52-4.75 (m, 3H), 5.44 (d, 1H, *J* = 16.0 Hz),

5.69 (d, 1H, *J* = 16.0 Hz), 7.03 (s, 1H), 7.14-7.17 (m, 1H), 7.03 (s, 1H), 7.69 (s, 2H), 8.45 (d, 1H, *J* = 4.4 Hz) and 9.11 (d, 1H, *J* = 5.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.4, 48.5, 55.7, 56.3, 56.5, 63.7, 108.2, 109.1, 110.8, 114.3, 117.6, 119.2, 127.6, 129.46, 129.49, 139.3, 142.8, 145.1, 147.9, 153.9, 156.4 and 170.4; mass spectrum (APCI), *m*/*z* 484.1467 $(M+H)^+$ ($C_{22}H_{22}N_5O_8$ requires *m*/*z* 484.1468).

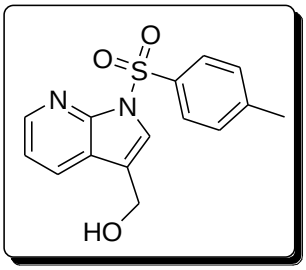


(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)-pdCpA (2.1). To a solution containing 5.20 mg (4.0 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 9.0 mg (21 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.19**). The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a reversed phase column (C_{18}, 10 × 250 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate (pH 4.5)–acetonitrile. The retention time of the desired product was 21 min. The fractions containing the product were lyophilized to afford **2.1** as a colorless solid: yield 1.61 mg (42%); mass spectrum (ESI), *m*/*z* 1061.2188 $(M-H)^-$ ($C_{39}H_{43}N_{12}O_{20}P_2$ requires *m*/*z* 1061.2192).

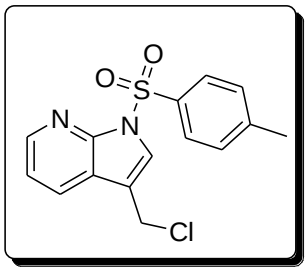


1-Tosyl-1*H*-pyrrolo[2,3-*b*]pyridine-3-carbaldehyde (2.21). To a stirred solution containing 1.00 g (8.47 mmol) of 1*H*-pyrrolo[2,3-*b*]pyridine in 10 mL of 1:1 AcOH–H_2O was added 1.78 g (12.7 mmol) of hexamethylenetetramine (HMTA). The reaction mixture was heated at reflux under argon overnight. The reaction mixture was cooled to 0 °C and the formed precipitate was filtered and dried under vacuum. 1*H*-pyrrolo[2,3-*b*]pyridine-3-carbaldehyde (**2.20**) was obtained as a colorless solid and was used directly in the next step without further purification. To a stirred solution containing 757 mg (5.17 mmol) of **2.20** in 20 mL of anhydrous DMF at 0 °C was added 414 mg (10.4 mmol) of NaH. The reaction mixture was stirred at 0 °C for 10 min under argon, and then 1.48 g (7.76 mmol) of *p*-TsCl was added. The reaction mixture was stirred at 0 °C under argon for 3 h, diluted with 100 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 4:1 hexanes–ethyl acetate gave 1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine-3-carbaldehyde (**2.21**) as a yellow solid: yield 1.34 g (53% for two steps); silica gel TLC R_f 0.35 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.40 (s, 3H), 7.27-7.34 (m, 3H), 8.16 (d, 2H, J = 8.4 Hz), 8.39 (s, 1H), 8.50 (d, 1H, J = 2.4 Hz), 8.51 (d, 1H, J = 5.6 Hz) and 10.04 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.8, 119.1,

119.5, 120.7, 120.8, 128.8, 130.1, 131.5, 134.4, 135.9, 146.5, 146.8 and 185.3; mass spectrum (APCI), *m*/*z* 301.0658 $(M+H)^+$ ($C_{15}H_{13}N_2O_3S$ requires *m*/*z* 301.0647).

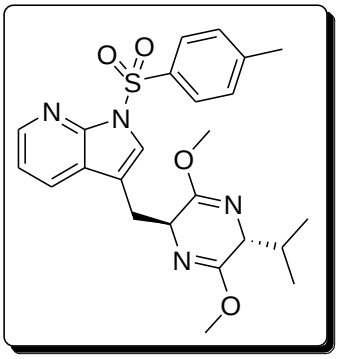


3-Hydroxymethyl-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine (2.22). To a suspension of 1.34 g (4.46 mmol) of 1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine-3-carbaldehyde (**2.21**) in 20 mL of EtOH was added 222 mg (8.92 mmol) of $NaBH_4$ and the reaction mixture was stirred at room temperature for 2 h, then diluted with 100 mL of satd aq $NaHCO_3$. The formed precipitate was filtered and dried under vacuum. 3-Hydroxymethyl-1-tosyl-*1H*-pyrrolo[2,3-*b*]pyridine (**2.22**) was obtained as an off-white solid: yield 1.20 g (90%); silica gel TLC R_f 0.1 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.29 (s, 3H), 4.75 (s, 2H), 7.10-7.20 (m, 3H), 7.62 (s, 1H), 7.88 (dd, 1H, *J* = 8.0 and 1.6 Hz), 7.99 (d, 2H, *J* = 8.0 Hz) and 8.37 (dd, 1H, *J* = 4.4 and 1.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 57.4, 118.8, 118.9, 122.0, 123.9 128.2, 128.7, 129.8, 135.5, 145.3, 145.4 and 147.8; mass spectrum (APCI), *m*/*z* 303.0805 $(M+H)^+$ ($C_{15}H_{15}N_2O_3S$ requires *m*/*z* 303.0803).



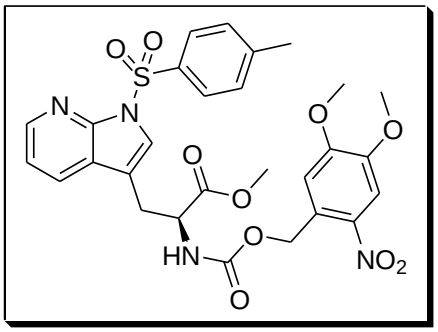
3-(Chloromethyl)-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine (2.23). To a cooled (⁻10 °C) solution containing 1.20 g (3.9 mmol) of 3-hydroxymethyl-1-tosyl-1*H*-pyrrolo[2,3-

b]pyridine (**2.22**) in 20 mL of anhydrous CH_2Cl_2 and 2.18 mL (1.58 g, 15.6 mmol) of Et_3N was added dropwise 0.58 mL (947 mg, 7.93 mmol) of $SOCl_2$. The reaction mixture was allowed to warm slowly to room temperature and was then stirred for 6 h, diluted with 50 mL of satd aq $NaHCO_3$, and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 4:1 hexanes–ethyl acetate gave 3-(chloromethyl)-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine (**2.23**) as a brown solid: yield 1.01 g (81%); silica gel TLC R_f 0.42 (4:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.33 (s, 3H), 4.69 (s, 2H), 7.18-7.25 (m, 3H), 7.74 (s, 1H), 7.92 (d, 1H, J = 1.2 Hz), 7.94 (d, 1H, J = 1.6 Hz), 8.06 (d, 1H, J = 8.0 Hz) and 8.43 (dd, 1H, J = 4.8 and 1.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 37.5, 15.4, 119.0, 121.5, 124.9, 128.2, 128.5, 129.8, 135.2, 145.5, 145.6 and 147.5; mass spectrum (APCI), *m*/*z* 321.0469 $(M+H)^+$ ($C_{15}H_{14}N_2O_2SCl$ requires *m*/*z* 321.0465).



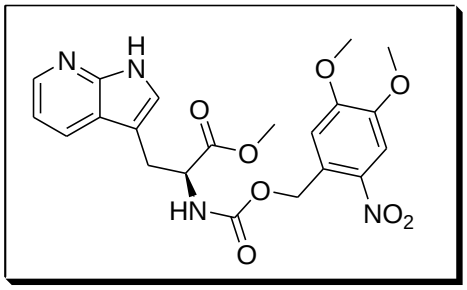
3-(((2*S*,5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine (2.24). To a stirred solution containing 0.62 mL (650 mg, 3.53 mmol) of Schöllkopf's reagent in 10 mL of anhydrous THF at ⁻78 °C was added 0.45 mL (308 mg, 4.82 mmol) of 2.5 M BuLi. The reaction mixture was stirred at ⁻78 °C for 30 min under argon and then a solution containing 1.03 g (3.21 mmol) of 3-(chloromethyl)-

1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine (**2.23**) in 10 mL of anhydrous THF was added. The reaction mixture was stirred for 1 h under argon at −78 °C, then diluted with 50 mL of satd aq NH_4Cl and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 2:1 hexanes–ethyl acetate gave 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine (**2.24**) as a yellow oil: yield 994 mg (66%); silica gel TLC R_f 0.15 (4:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.51(d, 3H, J = 6.8 Hz), 0.78 (d, 3H, J = 8.0 Hz), 1.99-2.03 (m, 1H), 2.22 (s, 3H), 3.07-3.12 (m, 3H), 3.57 (s, 3H), 3.60 (s, 3H), 4.22-4.25 (m, 1H), 7.02-7.05 (m, 1H), 7.13 (d, 1H, J = 8.0 Hz), 7.36 (s, 1H), 7.74 (dd, 1H, J = 8.0 and 1.6 Hz), 7.87 (d, 2H, J = 8.4 Hz) and 8.29 (dd, 1H, J = 4.8 and 1.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.4, 18.8, 21.5, 29.1, 31.3, 52.1, 52.3, 55.5, 60.4, 114.9, 118.3, 123.7, 124.6, 127.5, 128.2, 129.5, 135.5, 144.6, 144.8, 147.1, 161.8 and 164.0; mass spectrum (APCI), m/z 469.1921 $(M+H)^+$ ($C_{24}H_{29}N_4O_4S$ requires m/z 469.1909).

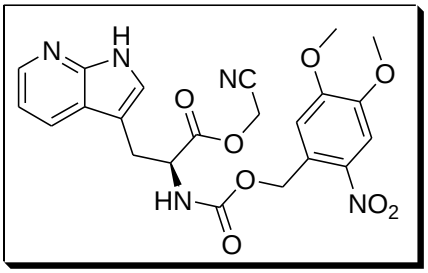


Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl) (2.26). To a stirred solution containing 783 mg (1.67 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-

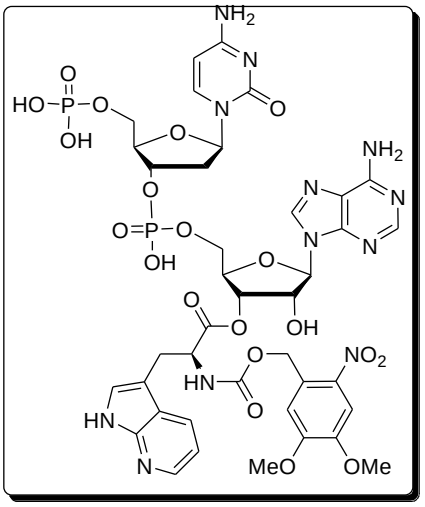
pyrrolo[2,3-*b*]pyridine (**2.24**) in 20 mL of THF at 0 °C was added 17.5 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then slowly poured into 50 mL of satd aq $NaHCO_3$ and then extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. Methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.25**) was obtained as a yellow oil and was used directly in the next step without further purification. To a stirred solution containing 450 mg (1.20 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.25**) in 4 mL of dioxane-water (1:1) was added 583 mg (4.22 mmol) of K_2CO_3 followed by 336 mg (1.56 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 12 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.26**) as a yellow oil: yield 588 mg (58% for two steps); silica gel TLC R_f 0.45 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.34 (s, 3H), 3.21-3.26 (m, 2H), 3.68 (s, 3H), 3.91 (s, 3H), 3.94 (s, 3H), 4.67-4.72 (m, 1H), 5.47-5.56 (m, 2H), 6.95 (s, 1H), 7.13-7.23 (m, 3H), 7.52 (s, 1H), 7.70 (s, 1H), 7.78 (d, 2H, J = 8.0 Hz), 8.01 (d, 2H, J = 8.4 Hz) and 8.40 (d, 1H, J = 4.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 28.1, 52.8, 54.1, 56.5, 56.6, 64.1, 108.3, 110.1, 113.4, 118.9, 123.0, 124.6, 127.7, 127.9, 128.0, 128.1,129.7, 135.4, 139.7, 145.4, 147.3, 148.3, 153.8, 155.3 and 171.6; mass spectrum (APCI), *m*/*z* 613.1613 $(M+H)^+$ ($C_{28}H_{29}N_4O_{10}S$ requires *m*/*z* 613.1604).



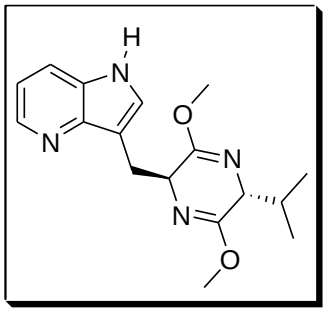
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (2.27). To a stirred solution containing 331 mg (0.54 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.26**) in 6 mL of 2:1 THF–methanol was added 572 mg (1.62 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 3 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.27**) as a yellow oil: 28.0 mg (15%); silica gel TLC R_f 0.2 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.27-3.28 (m, 1H), 3.65-3.66 (m, 1H), 3.70 (s, 3H), 3.73 (s, 3H), 3.88 (s, 3H), 4.76-4.80 (m, 1H), 5.44 (d, 1H, J = 15.2 Hz), 5.59 (d, 1H, J = 15.2 Hz), 6.47-6.51 (m, 1H), 6.93-7.01 (m, 3H), 7.65 (s, 1H), 7.78-7.82 (m, 1H), 8.21 (d, 1H, J =3.6 Hz) and 10.70 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.1, 52.5, 54.6, 56.1, 56.3, 63.8, 108.0, 109.8, 115.5, 120.1, 123.9, 127.1 128.2, 139.5, 142.5, 142.6, 148.0, 148.5, 153.6, 155.6 and 172.7; mass spectrum (APCI), *m*/*z* 459.1521 $(M+H)^+$ ($C_{21}H_{23}N_4O_8$ requires *m*/*z* 459.1516).



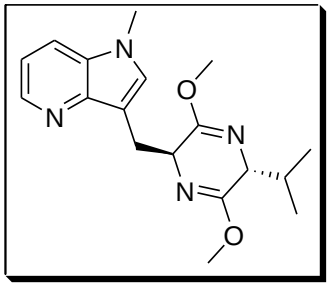
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (2.28). To a stirred solution containing 25.0 mg (0.05 mmol) of **2.27** in 1 mL of 1:3:1 water–THF–methanol was added 150 µL (0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 3 h, and then concentrated under diminished pressure. The residue was redissolved into 1 mL of anhydrous DMF and 23.0 µL (17.0 mg, 0.15 mmol) of Et_3N was added followed by 10.0 µL (11.2 mg, 0.15 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h and then diluted with 20 mL of EtOAc. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.28**) as a light yellow solid: yield 19.0 mg (73%); silica gel TLC R_f 0.45 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.33-3.34 (m, 1H), 3.69 (s, 1H), 3.80 (s, 3H), 3.92 (s, 3H), 4.67-4.88 (m, 3H), 5.47 (d, 1H, J = 14.8 Hz), 5.58 (d, 1H, J = 14.8 Hz), 6.25 (s, 1H), 6.93 (s, 1H), 7.00-7.03 (m, 1H), 7.15 (s, 1H), 7.67 (s, 1H), 7.82 (d, 1H, J = 7.6 Hz), 8.22 (d, 1H, J = 4.0 Hz) and 10.70 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.0, 49.2, 54.7, 56.5, 64.2, 107.5, 108.3, 110.3, 114.0, 116.0, 120.1, 124.1, 127.1, 127.7, 139.8, 143.1, 148.5, 153.7, 155.6 and 171.2; mass spectrum (APCI), *m*/*z* 484.1462 $(M+H)^+$ ($C_{22}H_{22}N_5O_8$ requires *m*/*z* 484.1468).



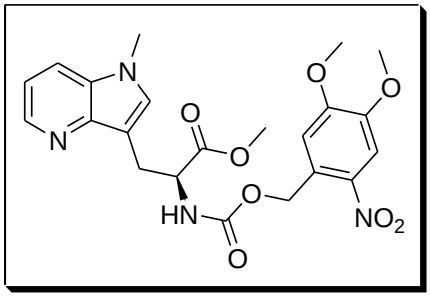
(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)-pdCpA (2.2). To a solution containing 5.20 mg (4.0 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 9.0 mg (21 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.28**). The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a reversed phase column (C_{18}, 10 × 250 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate (pH 4.5)–acetonitrile. The retention time of the desired product was 21 min. The fractions containing the product were lyophilized to afford **2.2** as a colorless solid: yield 2.50 mg (66%); mass spectrum (ESI), 1061.2185 $(M-H)^-$ ($C_{39}H_{43}N_{12}O_{20}P_2$ requires *m*/*z* 1061.2192).



3-(((2*S*, 5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1*H*-pyrrolo[3,2-*b*]pyridine (2.29). To a stirred solution containing 309 mg (0.66 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridine (**2.15**) in 5 mL of 2:1 THF–methanol was added 699 mg (1.98 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for overnight under argon. The reaction mixture was diluted with 20 mL of brine and extracted with portions of two 20-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with ethyl acetate gave the expected product 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1*H*-pyrrolo[3,2-*b*]pyridine (**2.29**) as a yellow oil: yield 141 mg (68%); silica gel TLC R_f 0.2 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.63 (d, 3H, J = 6.8 Hz), 0.94 (d, 3H, J = 7.2 Hz), 2.13-2.15 (m, 1H), 3.29-3.32 (m, 1H), 3.49-3.50 (m, 1H), 3.61 (s, 3H), 3.63 (s, 3H), 4.40-4.41 (m, 1H), 7.02-7.05 (m, 3H), 7.33 (s, 1H), 7.62 (d, 1H, J = 8.0 Hz), 8.45 (d, 1H, J = 4.4 Hz) and 10.19 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.7, 19.2, 28.6, 31.7, 52.4, 52.5, 56.5, 60.7, 112.5, 116.6, 118.2, 126.7, 128.8, 142.6, 145.9, 163.6 and 163.7; mass spectrum (APCI), *m*/*z* 315.1822 $(M+H)^+$ ($C_{17}H_{23}N_4O_2$ requires *m*/*z* 315.1821).

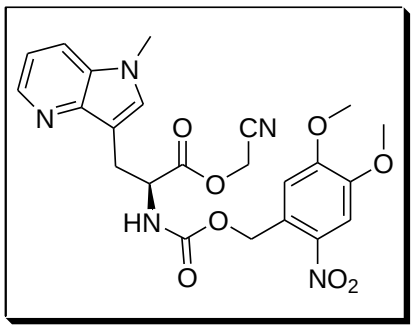


3-(((2*S*, 5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-methyl-1*H*-pyrrolo[3,2-*b*]pyridine (2.30). To a stirred solution containing 140 mg (0.45 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1*H*-pyrrolo[3,2-*b*]pyridine (**2.29**) in 3 mL of anhydrous DMF at 0 °C was added 21.4 mg (0.89 mmol) of NaH followed by 0.06 mL (127 mg, 0.89 mmol) of MeI. The reaction mixture was stirred at 0 °C under argon for 1 h. The reaction mixture was diluted with 100 mL of satd aq $NaHCO_3$, extracted with portions of two 20-mL portions of EtOAc and then dried under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave the expected product 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-methyl-1*H*-pyrrolo[3,2-*b*]pyridine (**2.30**) as a yellow oil: yield 108 mg (74%); silica gel TLC R_f 0.3 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.62 (d, 3H, J = 6.8 Hz), 0.95 (d, 3H, J = 6.8 Hz), 2.12-2.16 (m, 1H), 3.14-3.20 (m, 1H), 3.50-3.55 (m, 1H), 3.59-3.60 (m, 1H), 3.61 (s, 3H), 3.66 (s, 3H), 3.68 (s, 3H), 4.31-4.33 (m, 1H), 7.01-7.04 (m, 1H), 7.06 (s, 1H), 7.47 (d, 1H, J = 8.0 Hz) and 8.41 (d, 1H, J = 4.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.6, 19.1, 28.5, 31.7, 32.8, 52.3, 52.4, 56.5, 60.7, 111.7, 115.9, 116.0, 129.5, 130.5, 142.3, 146.1, 163.5 and 163.6; mass spectrum (APCI), *m*/*z* 329.1974 $(M+H)^+$ ($C_{18}H_{25}N_4O_2$ requires *m*/*z* 329.1977).



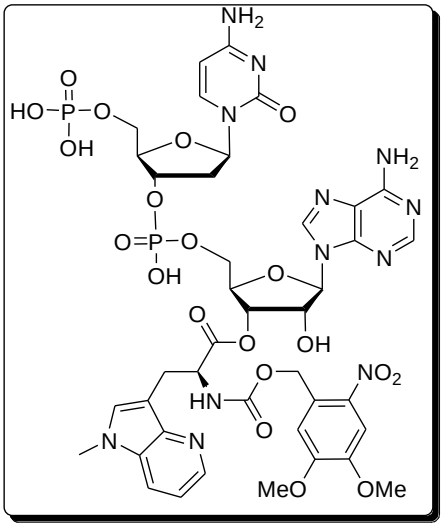
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (2.32). To a stirred solution containing 107 mg (0.33 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-methyl-1*H*-pyrrolo[3,2-*b*]pyridine (**2.30**) in 4 mL of THF at 0 °C was added 4 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then slowly poured into 50 mL of satd aq $NaHCO_3$ and then extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. Methyl (*S*)-2-amino-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.31**) was obtained as a yellow oil and was used directly in the next step without further purification. To a stirred solution containing 27.0 mg (0.12 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.31**) in 2 mL of dioxane-water (1:1) was added 56.0 mg (0.41 mmol) of K_2CO_3 followed by 39.1 mg (0.18 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 14 h under argon then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)- 2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.32**) as a yellow oil: yield 33.0 mg (25% over two steps); silica gel TLC R_f 0.1 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.29-3.31 (m, 2H), 3.60 (s, 3H), 3.69 (s, 3H), 3.72 (s, 3H), 3.84 (s, 3H), 4.55-4.56 (m, 1H), 5.38 (d, 1H, J = 16.0 Hz), 5.60 (d, 1H, J = 16 Hz), 6.99 (s, 1H), 7.06-7.09 (m, 2H), 7.55 (d, 1H, J = 8.0 Hz), 7.61 (s, 1H), 8.38 (d, 1H, J = 4.4 Hz) and 8.60 (d, 1H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ

27.0, 32.7, 51.9, 55.7, 56.1, 56.2, 63.1, 107.8, 108.9, 110.0, 116.6, 116.9, 129.5, 130.1, 131.2, 138.9, 141.9, 145.2, 147.5, 153.8, 156.1 and 171.9; mass spectrum (APCI), *m*/*z* 473.1682 $(M+H)^+$ ($C_{22}H_{25}N_4O_8$ requires *m*/*z* 473.1672).



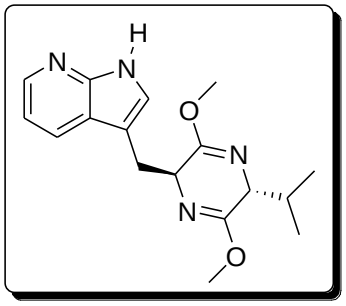
Cyanomethyl (*S*)- 2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (2.33). To a stirred solution containing 32.0 mg (0.07 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.32**) in 1 mL of 1:3:1 water–THF–methanol was added 214 µL (0.21 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 3 h, and then concentrated under diminished pressure. The residue was redissolved into 1 mL of anhydrous DMF and 30.0 µL (22.1 mg, 0.21mmol) of Et_3N was added followed by 13.0 µL (16.2 mg, 0.21 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h and then diluted with 20 mL of EtOAc. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.33**) as a light yellow solid: yield 18.0 mg (52%); silica gel TLC R_f 0.2 (1:1 hexanes–ethyl acetate); ^1H NMR ($CDCl_3$, 400 MHz) δ 3.42 (d, 2H, J = 4.4 Hz), 3.74 (s, 3H), 3.76

(s, 3H), 3.92 (s, 3H), 4.52-4.73 (m, 3H), 5.44 (d, 1H, *J* = 16 Hz), 5.69 (d, 1H, *J* = 16 Hz), 7.03 (s, 1H), 7.14-7.18 (m, 2H), 7.63 (dd, 1H, *J* =8.0 and 1.2 Hz), 7.69 (s, 1H) and 8.33 (dd, 1H, *J* = 4.8 and 1.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.9, 32.9, 48.4, 55.5, 56.3, 56.5, 63.6, 108.1, 109.1, 109.2, 114.4, 116.9, 117.3, 129.5, 130.5, 132.0, 139.2, 142.2, 145.3, 147.8, 153.9, 156.4 and 170.3; mass spectrum (APCI), *m*/*z* 498.1635 $(M+H)^+$ ($C_{23}H_{24}N_5O_8$ requires *m*/*z* 498.1625).



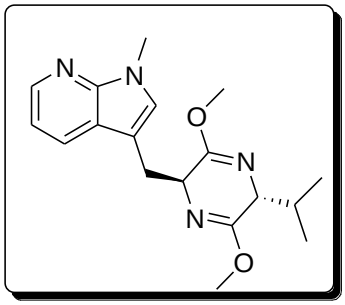
(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)-pdCpA (2.3). To a solution containing 5.20 mg (4.0 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 10.4 mg (21 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.33**). The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a reversed phase column (C_{18}, 10 × 250 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate (pH 4.5)–acetonitrile. The retention time of the desired product was 23.7 min. The fractions containing the product were lyophilized to

afford **2.3** as a colorless solid: yield 1.60 mg (39%); mass spectrum (ESI), 1075.2357 $(M-H)^-$ ($C_{40}H_{45}N_{12}O_{20}P_2$ requires *m*/*z* 1075.2348).



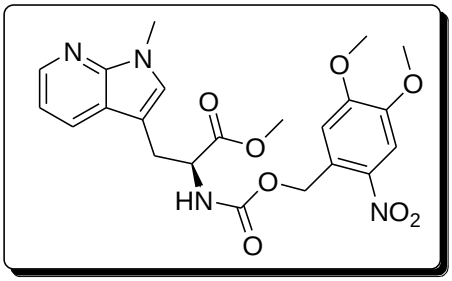
3-(((2*S*, 5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1*H*-pyrrolo[2,3-*b*]pyridine (2.34). To a stirred solution containing 303 mg (0.65 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine (**2.24**) in 5 mL of 2:1 THF–methanol was added 685 mg (1.94 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 12 h under argon, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1*H*-pyrrolo[2,3-*b*]pyridine (**2.34**) as a yellow oil: yield 77.0 mg (39%); silica gel TLC R_f 0.3 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.60 (d, 3H, *J* = 6.8 Hz), 0.91 (d, 3H, *J* = 6.8 Hz), 2.10-2.13 (m, 1H), 3.26-3.29 (m, 2H), 3.36-3.38 (m, 1H), 3.65 (s, 3H), 3.68 (s, 3H), 4.34-4.35 (m, 1H), 7.02 (dd, 1H, *J* = 4.4 and 4.4 Hz), 7.08 (s, 1H), 7.95 (d, 1H, *J* = 6.8 Hz), 8.26 (d, 1H, *J* = 4.8 Hz) and 11.52 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16 .5, 19.0, 29.7, 31.4, 52.3, 52.4, 56.7, 60.5,

109.8, 114.5, 121.3 123.9, 127.8, 142.0, 148.8, 162.8 and 163.9; mass spectrum (APCI), *m*/*z* 315.1829 $(M+H)^+$ ($C_{17}H_{23}N_4O_2$ requires *m*/*z* 315.1821).



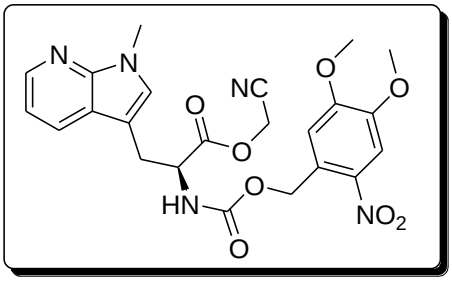
3-(((2*S*, 5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-methyl-1*H*-pyrrolo[2,3-*b*]pyridine (2.35). To a stirred solution containing 76.0 mg (0.24 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1*H*-pyrrolo[2,3-*b*]pyridine (**2.34**) in 3 mL of anhydrous DMF at 0 °C was added 11.5 mg (0.48 mmol) of NaH followed by 0.03 mL (69.0 mg, 0.48 mmol) of MeI. The reaction mixture was stirred at 0 °C under argon for 30 min, then diluted with 100 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-methyl-1*H*-pyrrolo[2,3-*b*]pyridine (**2.35**) as a yellow oil: yield 68.0 mg (83%); silica gel TLC R_f 0.4 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.59 (d, 3H, *J* = 6.8 Hz), 0.91 (d, 3H, *J* = 7.2 Hz), 2.09-2.13 (m, 1H), 3.22 (d, 2H, *J* = 4.4 Hz), 3.40-3.41 (m, 1H), 3.62 (s, 3H), 3.66 (s, 3H), 3.79 (s, 3H), 4.28-4.29 (m, 1H), 6.86 (s, 1H), 6.95-6.98 (m, 1H), 7.87 (dd, 1H, *J* = 8.0 and 1.6 Hz) and 8.25 (dd, 1H, *J* = 8.4 and 1.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.6, 19.1, 29.6, 31.1, 31.5, 52.2, 52.3, 56.7, 60.6, 108.9, 114.8,

121.3, 127.6, 127.7, 142.6, 147.7, 162.8 and 163.9; mass spectrum (APCI), *m*/*z* 329.1977 $(M+H)^+$ ($C_{18}H_{25}N_4O_2$ requires *m*/*z* 329.1977).



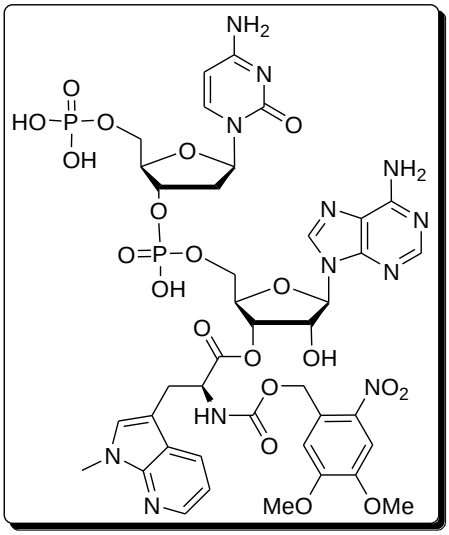
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (2.37). To a stirred solution containing 67.0 mg (0.21 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-methyl-1*H*-pyrrolo[2,3-*b*]pyridine (**2.35**) in 4 mL of THF at 0 °C was added 4 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then poured slowly into 30 mL of satd aq $NaHCO_3$ and then extracted with two 30-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. Methyl (*S*)-2-amino-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate was obtained as a yellow oil and was used directly in the next step without further purification. To a stirred solution containing 36.0 mg (0.15 mmol) of methyl (*S*)-2-amino-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.36**) in 2 mL of 1:1 dioxane–water was added 75.0 mg (0.54 mmol) of K_2CO_3 followed by 52.0 mg (0.24 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 14 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:3 hexanes–ethyl acetate gave methyl (*S*)-

2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.37**) as a yellow oil: yield 58.0 mg (62% for two steps); silica gel TLC R_f 0.3 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.24-3.26 (m, 2H), 3.66 (s, 3H), 3.78 (s, 3H), 3.83 (s, 3H), 3.88 (s, 3H), 4.65-4.67 (m, 1H), 5.46 (d, 1H, J = 8 Hz), 5.57 (d, 1H, J = 8.4 Hz), 6.88 (s, 1H), 6.96-6.99 (m, 2H), 7.63 (s, 1H), 7.76 (d, 1H, J = 7.2 Hz), 8.27 (dd, 1H, J = 4.8 and 1.2 Hz) and 8.60 (d, 1H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.9, 31.1, 52.5, 54.5, 56.4, 56.4, 63.9, 106.8, 108.1, 110.0, 115.3, 120.3, 126.8, 127.7, 127.8, 139.6, 143.1, 147.8, 148.0, 153.6, 155.3 and 172.2; mass spectrum (APCI), *m*/*z* 473.1673 $(M+H)^+$ ($C_{22}H_{25}N_4O_8$ requires *m*/*z* 473.1672).



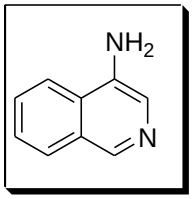
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (2.38). To a stirred solution containing 31.0 mg (0.07 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.37**) in 1 mL of 1:3:1 water–THF–methanol was added 214 µL (5.03 mg, 0.21 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 3 h, and then concentrated under diminished pressure. The residue was dissolved in 1 mL of anhydrous DMF and 30.0 µL (22.0 mg, 0.21 mmol) of Et_3N was added followed by 13.0 µL (16.0 mg, 0.21 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h, then diluted with 20 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The

combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.38**) as a light yellow solid: yield 22.1 mg (71%); silica gel TLC R_f 0.4 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.32 (d, 2H, J = 5.6 Hz), 3.84 (s, 3H), 3.89 (s, 3H), 3.93 (s, 3H), 4.62-4.81 (m, 3H), 5.43-5.55 (m, 2H), 6.90 (s, 1H), 7.04-7.07 (m, 2H), 7.69 (s, 1H), 7.83 (d, 1H, J = 7.6 Hz) and 8.33 (dd, 1H, J = 4.8 and 1.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.9, 31.3, 49.1, 54.6, 56.5, 56.6, 64.3, 105.9, 108.3, 110.3, 113.9, 115.7, 120.2, 126.8, 127.5, 128.1, 139.9, 143.5, 147.9, 148.4, 153.7, 155.4 and 170.7; mass spectrum (APCI), *m*/*z* 498.1638 $(M+H)^+$ ($C_{23}H_{24}N_5O_8$ requires *m*/*z* 498.1625).



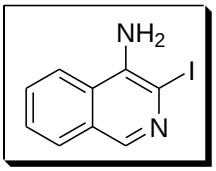
(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)pdCpA (3.4). To a solution containing 5.20 mg (4.00 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was

added 10.4 mg (21.0 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.38**). The reaction mixture was sonicated for 2 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate (pH 4.5) – acetonitrile. The retention time of the desired product was 23.4 min. The fractions containing the product were lyophilized to afford **2.4** as a colorless solid: yield 1.30 mg (31%); mass spectrum (ESI), 1075.2356 $(M-H)^-$ ($C_{40}H_{45}N_{12}O_{20}P_2$ requires *m*/*z* 1075.2348).

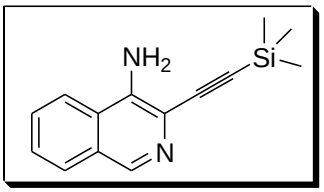


4-Aminoisoquinoline (2.39). To a stirred suspension containing 2.50 g (12.0 mmol) of 4-bromoisoquinoline in 20 mL of conc NH_4OH were added 38.0 mg (0.60 mmol) of Cu followed by 59.0 mg (0.60 mmol) of CuCl. The reaction mixture was heated at 100 °C in a high pressure tube for overnight. The cooled reaction mixture was diluted with 20 mL of water and extracted with three 40-mL portions of ethyl acetate. The combined organic solution was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 5 cm). Elution with 10:1 ethyl acetate–methanol gave the desired product **2.39** as a brown solid: yield 1.30 g (74%); mp 107-108 °C; silica gel TLC R_f 0.3 (10:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 500 MHz) δ 5.31 (br s, 2H), 7.26-7.29 (m, 1H), 7.37-7.40 (m, 1H), 7.58 (d, 1H, J = 8.0 Hz), 7.85-7.90 (m, 2H) and 8.36 (s, 1H); ^{13}C NMR (CD_3OD, 125 MHz)

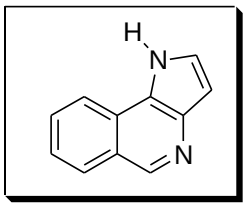
δ 121.7, 126.9, 127.0, 128.2, 128.4, 129.88, 129.91, 140.6 and 141.5; mass spectrum (APCI), *m*/*z* 145.0769 $(M+H)^+$ ($C_9H_9N_2$ requires *m*/*z* 145.0766).



4-Amino-3-iodoisoquinoline (2.40). To a stirred solution containing 1.76 g (6.94 mmol) of iodine in 60 mL of ethanol was added 2.60 g (8.33 mmol) of silver (I) sulfate. The reaction mixture was stirred at 23 °C for 5 min and then 1.00 g (6.94 mmol) of 4-aminoisoquinoline (**2.39**) was added. The reaction mixture was stirred in the dark at 23 °C for 16 h, then filtered and the filtrate was concentrated under diminished pressure. The residue was dissolved into 100 mL of ethyl acetate and washed with a satd aq solution of sodium thiosulfate. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 5 cm). Elution with 1:1 hexanes–ethyl acetate gave the desired product as a brown solid: yield 1.32 g (71%); mp 167-168 °C; silica gel TLC R_f 0.7 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.59 (br s, 2H), 7.59-7.69 (m, 2H), 7.76 (d, 1H, *J* = 8.8 Hz), 7.88 (d, 1H, *J* = 8.0 Hz) and 8.49 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 99.8, 120.6, 125.0, 127.8, 127.9, 128.5, 129.9, 138.6 and 142.9; mass spectrum (APCI), *m*/*z* 270.9734 $(M+H)^+$ ($C_9H_8N_2I$ requires *m*/*z* 270.9733).

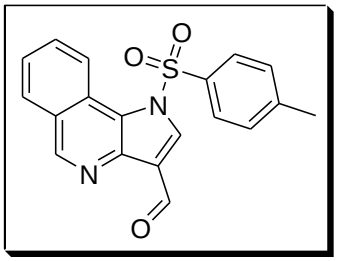


4-Amino-3-acetylenetrimethylsililisoquinoline (2.41). To a stirred solution containing 742 mg (2.75 mmol) of 4-amino-3-iodoisoquinoline (**2.40**) in 20 mL of degassed THF were added 27.0 mg (0.14 mmol) of CuI followed by 98.0 mg (0.14 mmol) of $Pd_2Cl_2(PPh_3)_2$, 1.52 mL (11.0 mmol) of triethylamine and 0.78 mL (5.50 mmol) of trimethylsilylacetylene (TMSA). The reaction mixture was stirred at 23 °C for 16 h, then filtered and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 4:1 hexanes–ethyl acetate gave the desired product as a brown solid: yield 602 mg (91%); silica gel TLC R_f 0.2 (4:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.22 (s, 9H), 4.98 (br s, 2H), 7.44-7.49 (m, 2H), 7.72-7.76 (m, 2H) and 8.53 (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ -0.02, 100.6, 101.7, 118.5, 120.5, 124.7, 127.7, 127.8, 128.2, 129.3, 140.6 and 142.2; mass spectrum (APCI), *m*/*z* 241.1160 $(M+H)^+$ ($C_{14}H_{17}N_2Si$ requires *m*/*z* 241.1161).



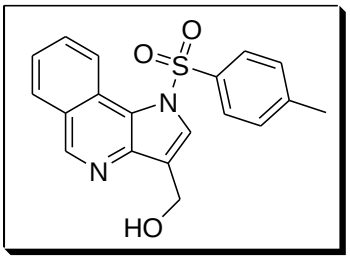
1*H*-Pyrrolo[3,2-*c*]isoquinoline (2.42). To a stirred solution containing 650 mg (2.70 mmol) of **2.41** in 25 mL of anhydrous DMF was added 316 mg (8.10 mmol) of sodium amide and the reaction mixture was heated at 100 °C for 16 h. The cooled reaction mixture was then poured into 50 mL of water and extracted with three 30-mL portions of of ethyl acetate. The combined organic solution was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with ethyl acetate gave the expected product as a yellow

solid: yield 355 mg (78%); silica gel TLC R_f 0.4 (10:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 6.65 (d, 1H, J = 3.2 Hz), 7.28-7.31 (m, 1H), 7.32 (d, 1H, J = 3.2 Hz), 7.53-7.57 (m, 1H), 7.85 (d, 1H, J = 8.4 Hz), 8.05 (d, 1H, J = 8.4 Hz) and 8.70 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 103.9, 120.7, 124.2, 125.6, 125.8, 126.1, 129.8, 130.0, 131.4, 140.2 and 146.6; mass spectrum (APCI), m/z 169.0763 $(M+H)^+$ ($C_{11}H_9N_2$ requires m/z 169.0766).



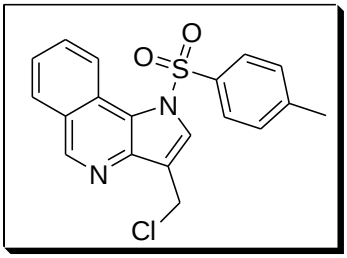
1-Tosyl-1*H*-pyrrolo[3,2-*c*]isoquinoline-3-carbaldehyde (2.44). To a stirred solution containing 719 mg (4.27 mmol) of 1*H*-pyrrolo[3,2-*c*]isoquinoline (**2.42**) in 7 mL of 1:1 AcOH–H_2O was added 900 mg (6.42 mmol) of hexamethylenetetramine (HMTA). The reaction mixture was heated at reflux under argon overnight. The cooled reaction mixture was diluted with 40 mL of water. The pH was adjusted to >12 and the reaction mixture was extracted with three 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. 1*H*-Pyrrolo[3,2-*c*]isoquinoline-3-carbaldehyde (**2.43**) was obtained as a light brown solid and was used directly in the next step without further purification. To a stirred solution containing 545 mg (2.78 mmol) of 1*H*-pyrrolo[3,2-*c*]isoquinoline-3-carbaldehyde (**2.43**) in 20 mL of anhydrous DMF at 0 °C was added 133 mg (5.56 mmol) of NaH. The reaction mixture was stirred at 0 °C for 10 min under argon and then 1.06 g (5.56 mmol) of *p*-TsCl was added. The reaction mixture was stirred at 0 °C under argon for 3 h, diluted with 100 mL of satd aq $NaHCO_3$

and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with ethyl acetate gave 1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinoline-3-carbaldehyde (**2.44**) as a yellow solid: yield 691 mg (46% for two steps); silica gel TLC R_f 0.65 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.25 (s, 3H), 7.16 (d, 2H, J = 8.4 Hz), 7.56 (t, 1H, J = 7.6 Hz), 7.64 (d, 2H, J = 8.4 Hz), 7.72-7.76 (m, 1H), 8.00 (d, 1H, J = 8.0 Hz), 8.62 (s, 1H), 8.95 (d, 1H, J = 8.8 Hz), 9.13 (s, 1H) and 10.50 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 121.3, 122.6, 123.5, 124.9, 126.5, 127.1, 127.2, 129.3, 130.4, 131.5, 134.1, 134.8, 140.7, 146.5, 152.5 and 185.6; mass spectrum (APCI), m/z 351.0811 $(M+H)^+$ ($C_{19}H_{15}N_2O_3S$ requires m/z 351.0803).



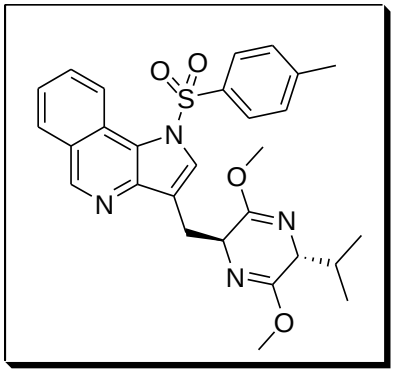
1-Tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)methanol (2.45). To a suspension of 690 mg (1.97 mmol) of 1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinoline-3-carbaldehyde (**2.44**) in 10 mL of EtOH was added 149 mg (3.94 mmol) of $NaBH_4$. The reaction mixture was stirred at room temperature for 2 h, diluted with 100 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with ethyl acetate gave 1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)methanol (**2.45**) as an off-white solid: yield 779 mg (96%); silica gel

TLC R_f 0.1 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ2.25 (s, 3H), 5.06 (s, 2H), 7.11 (d, 2H, J = 8.0 Hz), 7.53-7.56 (m, 1H), 7.59 (d, 2H, J = 8.4 Hz), 7.73-7.77 (m, 1H), 7.98 (s, 1H), 8.00 (s, 1H), 9.00 (s, 1H) and 9.05 (d, 1H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ21.7, 56.8, 122.9, 123.5, 125.7, 126.0, 126.8, 126.9, 128.6, 129.3, 130.1, 131.2, 132.1, 135.1, 143.1, 145.5 and 150.7; mass spectrum (APCI), m/z 353.0979 $(M+H)^+$ ($C_{19}H_{17}N_2O_3S$ requires m/z 353.0960).



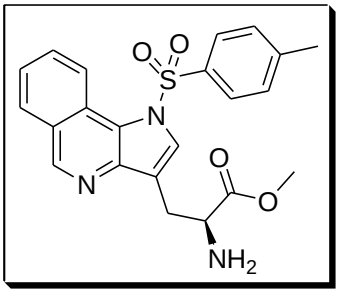
3-(Chloromethyl)-1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinoline (2.46). To a cooled (0 °C) solution containing 0.35 g (0.99 mmol) of 1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)methanol (**2.45**) in 10 mL of anhydrous CH_2Cl_2 and 0.25 mL (0.18 g, 1.79 mmol) of Et_3N was added dropwise 0.29 mL (177 mg, 1.5 mmol) of $SOCl_2$. The reaction was left to warm slowly to room temperature and stirred for 2 h, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 4:1 hexanes–ethyl acetate gave 3-(chloromethyl)-1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinoline (**2.46**) as a yellow oil: yield 283 mg (77%); silica gel TLC R_f 0.7 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ2.21 (s, 3H), 4.95 (s, 2H), 7.10 (d, 2H, J = 8.4 Hz), 7.52 (t, 1H, J = 7.6 Hz), 7.58 (d, 2H, J = 8.4 Hz), 7.71-7.75 (m, 1H), 7.97 (d, 1H, J = 8.4 Hz), 8.10 (s, 1H), 9.02 (d, 1H, J = 8.8 Hz) and 9.06 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ21.5,

35.9, 120.3, 122.7, 123.3, 125.4, 126.0, 126.81, 126.84, 128.7, 129.2, 130.1, 131.1, 134.8, 141.9, 145.6 and 151.2; mass spectrum (APCI), *m*/*z* 371.0621 $(M+H)^+$ ($C_{19}H_{16}N_2O_2ClS$ requires *m*/*z* 371.0621).



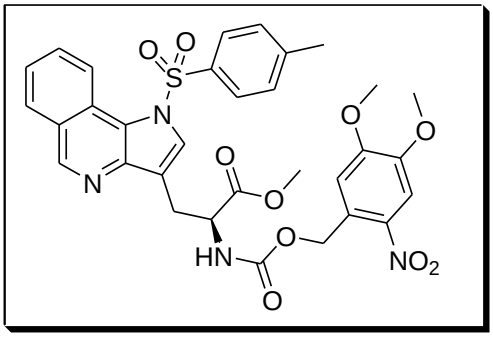
3-(((2*S*,5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinoline (2.47). To a stirred solution containing 0.15 mL (164 mg, 0.89 mmol) of Schöllkopf's reagent in 5 mL of anhydrous THF at ⁻78 °C was added 0.11 mL (78.0 mg, 1.22 mmol) of 2.5 M BuLi. The reaction mixture was stirred at ⁻78 °C for 30 min under argon and then a solution containing 301 mg (0.81 mmol) of 3-(chloromethyl)-1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinoline (**2.46**) in 5 mL of anhydrous THF was added. The reaction mixture was left to warm slowly to room temperature and stirred for 30 min under argon, then diluted with 50 mL of satd aq NH_4Cl and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 hexanes–ethyl acetate gave 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinoline (**2.47**) as a yellow oil: yield 251 mg (60%); silica gel TLC R_f 0.65 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.64 (d, 3H, J = 6.8 Hz), 0.93 (d,

3H, *J* = 6.8 Hz), 2.12-2.16 (m, 1H), 2.18 (s, 3H), 3.24-3.29 (m, 1H), 3.50-3.58 (m, 2H), 3.62 (s, 3H), 3.66 (s, 3H), 4.41-4.42 (m, 1H), 7.04 (d, 2H, *J* = 8.4 Hz), 7.44-7.54 (m, 3H), 7.66-7.70 (m, 1H), 7.82 (s, 1H), 7.93 (d, 1H, *J* = 8.0 Hz), 9.01 (s, 1H) and 9.04 (t, 1H, *J* = 8.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.0, 19.1, 21.5, 28.5, 31.8, 52.4, 52.5, 55.5, 60.8, 120.0, 122.7, 122.8, 125.5, 125.6, 126.6, 126.7, 128.4, 129.0, 129.9, 130.8, 135.3, 144.4, 145.0, 150.6, 163.0 and 163.8; mass spectrum (APCI), *m*/*z* 519.2067 $(M+H)^+$ ($C_{28}H_{31}N_4O_4S$ requires *m*/*z* 519.2066).



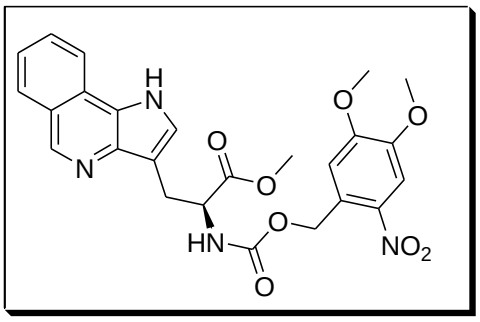
Methyl (*S*)-2-Amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (2.48). To a stirred solution containing 251 mg (0.48 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinoline (**2.47**) in 10 mL of THF at 0 °C was added 7 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then slowly poured into 50 mL of satd aq $NaHCO_3$ and then extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.48**) as a yellow oil: yield 150 mg (73%); silica gel TLC R_f 0.45 (1:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 2.24 (s, 3H), 3.24-3.29 (m, 1H), 3.33-3.38 (m, 1H), 3.68 (s, 3H), 4.04 (t, 1H, *J* = 6.4 Hz), 7.19 (d, 2H, *J* =

8.4 Hz), 7.56-7.60 (m, 3H), 7.76-7.80 (m, 1H), 7.98 (s, 1H), 8.09 (d, 1H, J = 8.0 Hz) and 9.04-9.07 (m, 2H); ^{13}C NMR (CD_3OD, 100 MHz) δ 21.5, 30.0, 52.7, 55.2, 120.2, 123.9, 124.6, 126.8, 127.3, 128.0, 128.1, 130.1, 130.5, 131.2, 132.4, 136.2, 144.5, 147.2, 151.9 and 175.8; mass spectrum (APCI), m/z 424.1327 $(M+H)^+$ ($C_{22}H_{22}N_3O_4S$ requires m/z 424.1331).



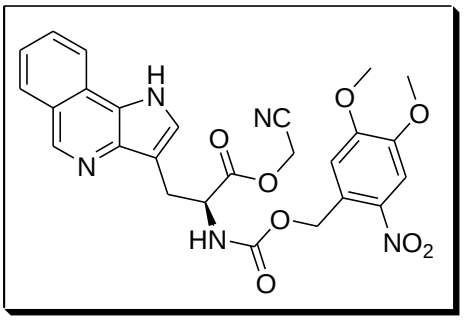
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (2.49). To a stirred solution containing 88.0 mg (0.21 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.48**) in 1 mL of 1:1 dioxane–water was added 101 mg (0.73 mmol) of K_2CO_3 followed by 59.0 mg (0.28 mmol) of NVOCCl. The reaction mixture was stirred at room temperature for 12 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.49**) as a yellow oil: yield 123 mg (90%); silica gel TLC R_f 0.45 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.23 (s, 3H), 3.42-3.43 (m, 2H), 3.59 (s, 3H), 3.68 (s, 3H), 3.87 (s, 3H), 4.68-4.70 (m, 1H), 5.49 (ABq, 2H,

J = 16 Hz), 6.90 (s, 1H), 7.11 (d, 2H, *J* = 8.4 Hz), 7.51-7.55 (m, 3H), 7.63 (s, 1H), 7.73-7.74 (m, 2H), 7.87 (s, 1H), 7.97 (d, 1H, *J* = 8.0 Hz) and 9.01-9.04 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.5, 26.8, 52.3, 55.0, 56.1. 56.3, 63.5, 108.0, 109.2, 118.4, 122.8, 123.4, 125.5, 126.1, 126.7, 128.5, 129.0, 129.15, 129.24, 130.1, 131.4, 134.9, 139.2, 143.0, 145.6, 147.7, 150.5, 153,7, 155.9 and 171.7; mass spectrum (APCI), *m*/*z* 663.1765 $(M+H)^+$ ($C_{32}H_{31}N_4O_{10}S$ requires *m*/*z* 663.1760).



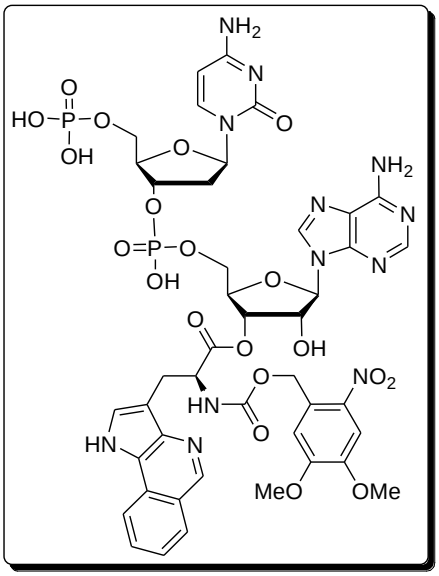
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (2.50). To a stirred solution containing 123 mg (0.19 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.49**) in 3 mL of 2:1 THF–methanol was added 197 mg (0.56 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 2 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.50**) as a yellow solid: yield 69.1 mg (73%); silica gel TLC R_f 0.40 (1:1 hexanes–ethyl acetate); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.19-3.23 (m, 1H), 3.38-3.41 (m, 1H), 3.61 (s,

3H), 3.74 (s, 3H), 3.85 (s, 3H), 4.53-4.57 (m, 1H), 5.34 (ABq, 2H, J = 15.0 Hz), 7.09 (s, 1H), 7.43 (br s, 1H), 7.54 (t, 1H, J = 7.5 Hz), 7.68 (s, 1H), 7.79 (t, 1H, J = 7.5 Hz), 8.14 (d, 1H, J = 8.0 Hz), 8.31 (d, 1H, J = 8.5 Hz), 8.39 (d, 1H, J = 7.5 Hz) and 8.97 (s, 1H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ26.4, 51.8, 54.9, 55.8, 56.0, 62.4, 108.0, 109.8, 111.6, 119.9, 122.1, 123.5, 124.1, 124.4, 124.5, 127.9, 128.5, 129.9, 138.2, 138.9, 145.1, 147.5, 153.3, 155.4 and 172.3; mass spectrum (APCI), m/z 509.1682 $(M+H)^+$ ($C_{25}H_{25}N_4O_8$ requires m/z 509.1672).



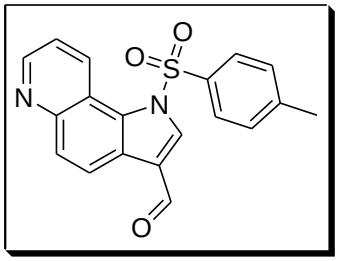
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (2.51). To a stirred solution containing 26.0 mg (0.05 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.50**) in 1 mL of 1:3:1 water–THF–methanol was added 150 µL (0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, then concentrated under diminished pressure. The residue was redissolved in 1 mL of anhydrous DMF under argon. To the stirred solution was added 20 µL (15.0 mg, 0.15 mmol) of Et_3N followed by 10.0 µL (11.0 mg, 0.15 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h and then diluted with 20 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure.

The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.51**) as a light yellow solid: yield 14.0 mg (54%); silica gel TLC R_f 0.4 (1:1 hexanes–ethyl acetate); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.24-3.30 (m, 1H), 3.38-3.43 (m, 1H), 3.75 (s, 3H), 3.85 (s, 3H), 4.64-4.69 (m, 1H), 4.99 (s, 2H), 5.36 (ABq, 2H, J = 15.2 Hz), 7.10 (s, 1H), 7.43-7.44 (m, 1H), 7.55 (t, 1H, J = 7.6 Hz), 7.69 (s, 1H), 7.78-7.82 (m, 1H), 8.14-8.16 (m, 1H), 8.32 (d, 1H, J = 8.4 Hz), 8.53 (d, 1H, J = 7.6 Hz) and 8.98 (s, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 26.3, 49.3, 54.6, 55.9, 56.0, 62.5, 104.5, 108.1, 109.9, 110.9, 115.6, 119.9, 122.2, 123.7, 124.1, 124.5, 127.7, 128.5, 129.9, 138.1, 139.0, 145.2, 147.6, 153.3, 155.4 and 171.0; mass spectrum (APCI), *m*/*z* 534.1634 $(M+H)^+$ ($C_{26}H_{24}N_5O_8$ requires *m*/*z* 534.1625).



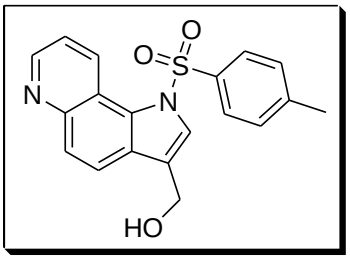
(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)-pdCpA (2.5). To a solution containing 5.20 mg (4.0 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 11.0

mg (21 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.51**). The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a reversed phase column (C_{18}, 10 × 250 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate (pH 4.5)–acetonitrile. The retention time of the desired product was 27.2 min. The fractions containing the product were lyophilized to afford **2.5** as a colorless solid: yield 2.21 mg (55%); mass spectrum (ESI), *m*/*z* 1111.2344 $(M-H)^-$ ($C_{43}H_{45}N_{12}O_{20}P_2$ requires *m*/*z* 1111.2348).



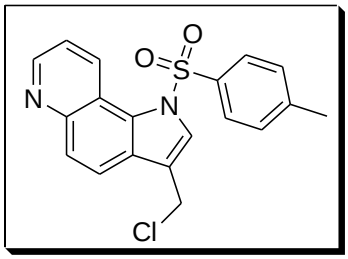
1-Tosyl-1*H*-pyrrolo[2,3-*f*]quinoline-3-carbaldehyde (2.53). To a stirred solution containing 600 mg (3.59 mmol) of 1*H*-pyrrolo[2,3-*f*]quinoline in 7 mL of 1:1 AcOH–H_2O was added 754 mg (5.34 mmol) of HMTA. The reaction mixture was heated at reflux under argon overnight. The cooled reaction mixture was diluted with 40 mL of water. The pH was adjusted to >12 and the reaction mixture was extracted with three 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. 1*H*-pyrrolo[2,3-*f*]quinoline-3-carbaldehyde (**2.52**) was obtained as a light brown solid and was used directly in the next step without further purification. To a stirred solution containing 545 mg (2.78 mmol) of 1*H*-pyrrolo[2,3-*f*]quinoline-3-carbaldehyde (**2.52**) in 20 mL of anhydrous DMF at 0 °C was added 133 mg (5.56 mmol) of NaH. The reaction mixture was stirred at 0 °C for 10 min

under argon and then 1.06 g (5.56 mmol) of *p*-TsCl was added. The reaction mixture was stirred at 0 °C under argon for 3 h, diluted with 100 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave 1-tosyl-1*H*-pyrrolo[2,3-*f*]quinoline-3-carbaldehyde (**2.53**) as a colorless solid: yield 691 mg (55% for two steps); silica gel TLC R_f 0.6 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.20 (s, 3H), 7.11 (d, 2H, *J* = 8.4 Hz), 7.37 (dd, 1H, *J* = 8.8 and 4.4 Hz), 7.56 (d, 2H, *J* = 8.4 Hz), 7.93 (d, 1H, *J* = 8.8 Hz), 8.49 (d, 1H, *J* = 8.8 Hz), 8.57 (s, 1H), 8.78 (dd, 1H, *J* = 4.4 and 1.6 Hz), 9.28 (d, 1H, *J* = 8.8 Hz) and 10.11 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.5, 117.7, 121.0, 121.5, 123.3, 125.4, 127.0, 128.6, 130.0, 130.4, 131.6, 133.8, 139.3, 146.4, 147.5, 149.0 and 185.6; mass spectrum (APCI), *m*/*z* 351.0812 $(M+H)^+$ ($C_{19}H_{15}N_2O_3S$ requires *m*/*z* 351.0803).



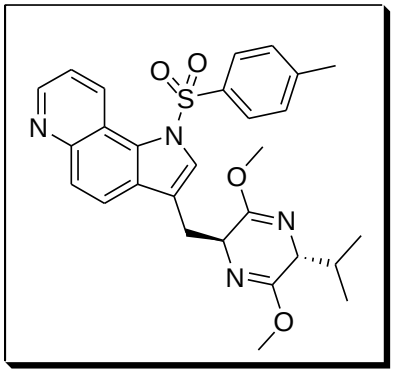
1-Tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)methanol (2.54). To a suspension of 690 mg (1.97 mmol) of 1-tosyl-1*H*-pyrrolo[2,3-*f*]quinoline-3-carbaldehyde (**2.53**) in 10 mL of EtOH was added 149 mg (3.94 mmol) of $NaBH_4$. The reaction mixture was stirred at room temperature for 2 h, diluted with 100 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel

column (10 × 4 cm). Elution with ethyl acetate gave 1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)methanol (**2.54**) as an off-white solid: yield 569 mg (82%); silica gel TLC R_f 0.25 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.22 (s, 3H), 4.83 (s, 2H), 7.14 (d, 2H, J = 8.0 Hz), 7.49-7.53 (m, 3H), 7.84 (d, 1H, J = 8.8 Hz), 7.95-7.97 (m, 2H), 8.72 (d, 1H, J = 4.4 Hz) and 9.52 (d, 1H, J = 8.4 Hz); ^{13}C NMR (5:1 CD_3OD-$CDCl_3$, 100 MHz) δ 21.5, 56.6, 120.0, 121.9, 123.6, 124.3, 126.6, 127.8, 129.4, 130.2, 130.9, 131.0, 134.0, 135.8, 146.9, 147.5 and 148.9; mass spectrum (APCI), *m*/*z* 353.0965 $(M+H)^+$ ($C_{19}H_{17}N_2O_3S$ requires *m*/*z* 353.0960).



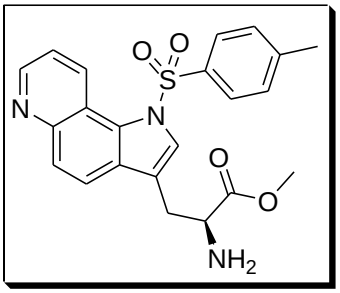
3-(Chloromethyl)-1-tosyl-1*H*-pyrrolo[2,3-*f*]quinoline (2.55). To a cooled (0 °C) solution containing 0.35 g (0.99 mmol) of 1-tosyl-1*H*-pyrrolo[2,3-*f*] quinolin-3-yl)methanol (**2.54**) in 10 mL of anhydrous CH_2Cl_2 and 0.25 mL (0.18 g, 1.79 mmol) of Et_3N was added dropwise 0.29 mL (177 mg, 1.5 mmol) of $SOCl_2$. The reaction mixture was allowed to warm slowly to room temperature and stirred for 2 h. The reaction mixture was then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 4:1 hexanes–ethyl acetate gave 3-(chloromethyl)-1-tosyl-1*H*-pyrrolo[2,3-*f*] quinoline (**2.55**) as a yellow oil: yield 226 mg (62%); silica gel TLC R_f 0.55 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.01 (s, 3H), 4.72 (s,

2H), 6.92 (d, 2H, *J* = 8.4 Hz), 7.32 (dd, 1H, *J* = 8.8 and 4.4 Hz), 7.42 (d, 2H, *J* = 8.4 Hz), 7.77 (d, 1H, *J* = 9.2 Hz), 7.86 (d, 1H, *J* = 9.2 Hz), 7.92 (s, 1H), 8.72 (d, 1H, *J* = 4.4 Hz) and 9.32 (d, 1H, *J* = 8.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.2, 36.8, 118.9, 120.7, 121.1, 126.5, 127.1, 127.7, 128.8, 129.7, 129.8, 131.6, 134.3, 145.3, 147.0 and 148.4; mass spectrum (APCI), *m*/*z* 371.0621 $(M+H)^+$ ($C_{19}H_{16}N_2O_2ClS$ requires *m*/*z* 371.0621).



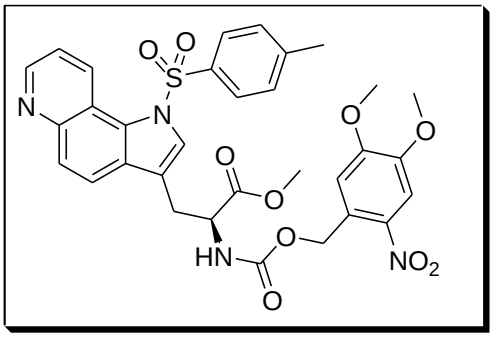
3-(((2*S*,5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[2,3-*f*]quinoline (2.56). To a stirred solution containing 0.15 mL (164 mg, 0.89 mmol) of Schöllkopf's reagent in 5 mL of anhydrous THF at −78 °C was added 0.11 mL (78.0 mg, 1.22 mmol) of 2.5 M BuLi. The reaction mixture was stirred at −78 °C for 30 min under argon and then a solution containing 300 mg (0.81 mmol) of 3-(chloromethyl)-1-tosyl-1*H*-pyrrolo[2,3-*f*]quinoline (**2.55**) in 5 mL of anhydrous THF was added. The reaction mixture was allowed to warm slowly to room temperature and stirred under argon for 30 min. The reaction mixture was then diluted with 50 mL of satd aq NH_4Cl and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 hexanes–ethyl acetate gave 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[2,3-*f*]quinoline (**2.56**) as a yellow oil: yield 312 mg

(66%); silica gel TLC R_f 0.5 (1:2 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.62 (d, 3H, J = 6.8 Hz), 0.91 (d, 3H, J = 6.8 Hz), 2.10-2.14 (m, 1H), 2.21 (s, 3H), 3.29-3.31 (m, 2H), 3.39-3.41 (m, 1H), 3.65 (s, 3H), 3.69 (s, 3H), 4.36-4.39 (m, 1H), 7.04 (d, 2H, J = 8.0 Hz), 7.36-7.42 (m, 3H), 7.68 (s, 1H), 7.87 (s, 2H), 8.78 (d, 1H, J = 4.4 Hz) and 9.46 (d, 1H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.6, 19.1, 21.6, 28.9, 31.7, 52.4, 52.5, 56.0, 60.7, 118.7, 118.9, 120.7, 122.5, 126.4, 126.6, 129.0, 129.3, 129.9, 130.6, 132.0, 135.0, 145.1, 147.2, 148.2, 162.3 and 164.1; mass spectrum (APCI), *m*/*z* 519.2059 $(M+H)^+$ ($C_{28}H_{31}N_4O_4S$ requires *m*/*z* 519.2066).



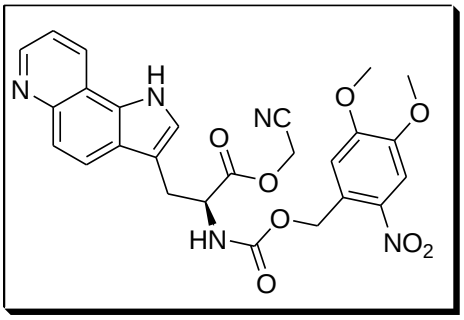
Methyl (*S*)-2-Amino-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (2.57). To a stirred solution containing 249 mg (0.47 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-pyrrolo[2,3-*f*]quinoline (**2.56**) in 10 mL of THF at 0 °C was added 7 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h, slowly poured into 50 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.57**) as a yellow oil: yield 174 mg (84%); silica gel TLC R_f 0.35 (1:1 ethyl acetate–methanol); ^{1}H NMR

(CD_3OD, 500 MHz) δ 2.26 (s, 3H), 3.17-3.27 (m, 2H), 3.64 (s, 3H), 3.89 (t, 1H, *J* = 6.5 Hz), 7.19 (d, 2H, *J* = 8.5 Hz), 7.53 (d, 2H, *J* = 8.5 Hz), 7.55-7.56 (m, 1H), 7.87-7.94 (m, 3H), 8.75 (dd, 1H, *J* = 4.5 Hz, *J* = 1.5 Hz) and 9.54 (d, 1H, *J* = 9.0 Hz); ^{13}C NMR (CD_3OD, 125 MHz) δ 21.5, 30.4, 52.7, 55.5, 111.5, 119.9, 120.3, 122.2, 123.2, 126.9, 128.0, 130.6, 131.2, 131.4, 134.2, 135.9, 147.3, 147.8, 149.3 and 175.8; mass spectrum (APCI), *m*/*z* 424.1337 $(M+H)^+$ ($C_{22}H_{22}N_3O_4S$ requires *m*/*z* 424.1331).



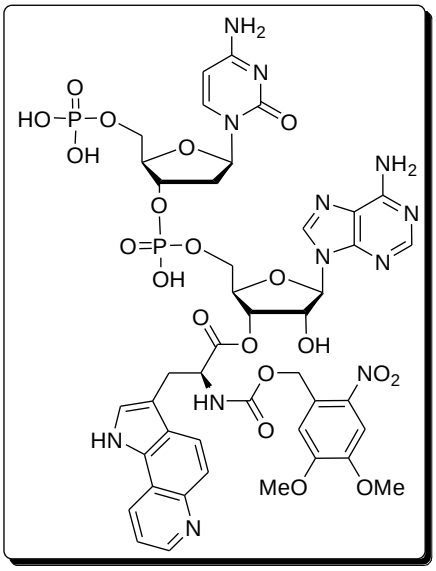
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (2.58). To a stirred solution containing 88.0 mg (0.21 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.57**) in 1 mL of dioxane–water was added 101 mg (0.73 mmol) of K_2CO_3 followed by 59.0 mg (0.28 mmol) of NVOCCl. The reaction mixture was stirred at room temperature for 12 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.58**) as a yellow oil: yield 126 mg (92%); silica gel TLC R_f 0.4 (1:1 hexanes–ethyl acetate); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.16 (s, 3H), 3.24-3.30 (m, 1H),

3.33-3.38 (m, 1H), 3.68 (s, 3H), 3.72 (s, 3H), 3.85 (s, 3H), 4.75-4.77 (m, 1H), 5.47 (ABq, 2H, *J* = 15.2 Hz), 6.12 (d, 1H, *J* = 8.0 Hz), 6.89 (s, 1H), 7.02 (d, 2H, *J* = 8.4 Hz), 7.33-7.40 (m, 3H), 7.60 (s, 1H), 7.68 (d, 1H, *J* = 9.2 Hz), 7.74 (s, 1H), 7.82 (d, 1H, *J* = 8.8 Hz), 8.71-8.72 (m, 1H) and 9.33 (d, 1H, *J* = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.4, 27.4, 52.7, 54.3, 56.25, 56.29, 63.9, 105.0, 108.0, 109.7, 117.3, 118.6, 120.9, 121.0, 126.6, 127.1, 127.8, 128.6, 129.5, 129.9, 131.9, 134.5, 139.4, 145.4, 147.0, 148.0, 148.3, 153.6, 155.5 and 171.8; mass spectrum (APCI), *m*/*z* 663.1770 $(M+H)^+$ ($C_{32}H_{31}N_4O_{10}S$ requires *m*/*z* 663.1760).

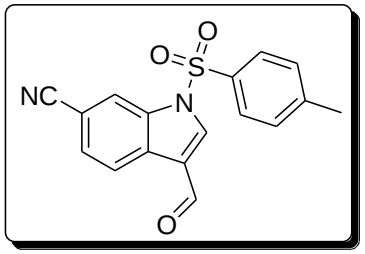


Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (2.60). To a stirred solution containing 120 mg (0.18 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.58**) in 3 mL of 2:1 THF–methanol was added 197 mg (0.56 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 3 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. Methyl (S)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.59**) was obtained as a yellow solid and was used directly in the next step without further

purification. Mass spectrum (APCI), *m*/*z* 509.1665$(M+H)^+$ ($C_{25}H_{25}N_4O_8$ requires *m*/*z* 509.1672). To a stirred solution containing 25.1 mg (50.0 μmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.59**) in 1 mL of 1:3:1 water–THF–methanol was added 150 μL (0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, then concentrated under diminished pressure. The residue was dissolved in 1 mL of anhydrous DMF under argon. To the stirred solution was added 21.0 μL (15.0 mg, 0.15 mmol) of Et_3N followed by 10.0 μL (11.0 mg, 0.15 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h and then diluted with 20 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with ethyl acetate gave cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.60**) as a light yellow solid: yield 14.0 mg (40%, for two steps); silica gel TLC R_f 0.35 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.41-3.46 (m, 1H), 3.69 (s, 2H), 3.83 (s, 3H), 3.92 (s, 3H), 4.61-4.67 (m, 1H), 4.74-4.88 (m, 1H), 5.45-5.57 (m, 2H), 6.89 (s, 1H), 7.19-7.21 (m, 1H), 7.41-7.44 (m, 1H), 7.66 (s, 1H), 7.78-7.86 (m, 2H), 8.33 (d, 1H, J = 8.0 Hz), 8.85 (d, 1H, J = 4.4 Hz) and 9.37 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.7, 49.1, 49.2, 54.9, 56.5, 64.4, 108.3, 110.2, 110.9, 114.1, 117.2, 120.5, 120.5, 121.8, 122.0, 122.7, 127.6, 128.3, 130.1, 139.7, 146.2, 147.8, 148.3, 153.7, 155.7 and 170.9; mass spectrum (APCI), *m*/*z* 534.1630 $(M+H)^+$ ($C_{26}H_{24}N_5O_8$ requires *m*/*z* 534.1625).

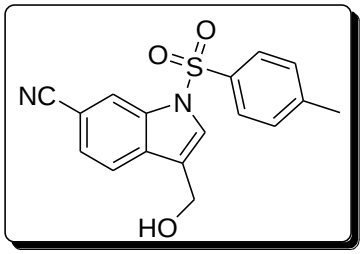


(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)-pdCpA (2.6). To a solution containing 5.20 mg (4.0 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 11.1 mg (21.0 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.60**). The reaction mixture was sonicated for 2 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (10 × 250 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate (pH 4.5)–acetonitrile. The retention time of the desired product was 23.9 min. The fractions containing the product were lyophilized to afford **2.6** as a colorless solid: yield 2.10 mg (54%); mass spectrum (ESI), *m*/*z* 1111.2344 $(M-H)^-$ ($C_{43}H_{45}N_{12}O_{20}P_2$ requires *m*/*z* 1111.2348).

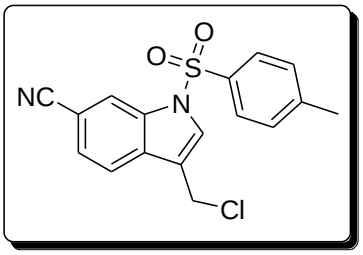


3-Formyl-1-tosyl-1*H*-indole-6-carbonitrile (2.62). Oxalyl chloride (0.60 mL, 0.89 g, 7.00 mmol) was added in a dropwise manner to 3 mL of cooled (ice bath) DMF with stirring. The reaction mixture was then stirred at 0 ^{o}C for 1 h. A solution of 1.00 g (7.03 mmol) of 6-cyanoindole in 3 mL of DMF was then added to the reaction mixture in a dropwise manner. The resulting reaction mixture was stirred at room temperature for overnight and 2 mL of 2 N NaOH was then added. The reaction mixture was heated at 100 ^{o}C for 30 min. The cooled reaction mixture was diluted with 50 mL of brine and then extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure to give 3-formyl-1*H*-indole-6-carbonitrile (**2.61**) as a yellow solid which was used directly in the next step without further purification. To a stirred solution containing 956 mg (5.62 mmol) of **2.61** in 20 mL of anhydrous DMF at 0 °C was added 450 mg (11.2 mmol) of NaH (60% suspension in mineral oil). The reaction mixture was stirred at 0 °C for 10 min under argon and then 1.50 g (7.86 mmol) of *p*TsCl was added. The reaction mixture was stirred at 0 °C under argon for 3 h, diluted with 150 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 4:1 hexanes–ethyl acetate gave the desired product **2.62** as a yellow solid: yield 1.55 g (68% for two steps); silica gel TLC R_f 0.82 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.37 (s, 3H), 7.33 (d, 2H, J = 8.4 Hz), 7.56 (d, 1H, J = 8.4 Hz), 7.86 (d, 2H, J = 8.4 Hz), 8.26 (s, 1H), 8.31 (d, 1H, J = 8.4 Hz), 8.42 (s, 1H) and 10.10 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 109.4, 117.6, 118.9, 121.6, 123.6, 127.3, 128.0, 129.5, 130.7,

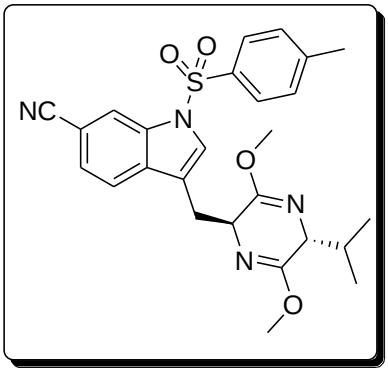
133.7, 134.1, 138.5, 147.0 and 184.9; mass spectrum (APCI), *m*/*z* 325.0650 $(M+H)^+$ ($C_{17}H_{13}N_2SO_3$ requires *m*/*z* 325.0647).



3-(Hydroxymethyl)-1-tosyl-1*H*-indole-6-carbonitrile (2.63). To a suspension of 1.29 g (4.46 mmol) of 3-formyl-1-tosyl-1*H*-indole-6-carbonitrile (**2.62**) in 15 mL of EtOH was added 339 mg (8.92 mmol) of $NaBH_4$ and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with 100 mL of satd aq $NaHCO_3$ and the formed precipitate was filtered and dried under vacuum. 3-(Hydroxymethyl)-1-tosyl-1*H*-indole-6-carbonitrile (**2.63**) was obtained as an off-white solid: yield 1.11 g (80%); silica gel TLC R_f 0.41 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.27 (s, 3H), 4.68 (s, 2H), 7.18 (d, 2H, *J* = 8.0 Hz), 7.62 (dd, 1H, *J* = 8.0 and 1.6 Hz), 7.62 (d, 1H, *J* = 8.4 Hz), 7.64 (s, 1H), 7.69 (d, 2H, *J* = 8.8 Hz) and 8.20 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.4, 55.8, 107.4, 117.8, 119.4, 121.1, 122.7, 126.2, 126.8, 126.9, 130.2, 132.9, 134.2, 134.5 and 145.8; mass spectrum (GCMS), *m*/*z* 326.0711 $(M)^+$ ($C_{17}H_{14}N_2SO_3$ requires *m*/*z* 326.0725).

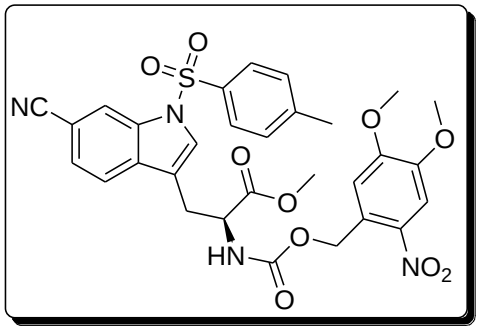


3-(Chloromethyl)-1-tosyl-1*H*-indole-6-carbonitrile (2.64). To a cooled (−10 °C) solution containing 1.11 g (3.40 mmol) of **2.63** in 20 mL of anhydrous CH_2Cl_2 and 2.18 mL (1.58 g, 15.6 mmol) of Et_3N was added dropwise 0.58 mL (947 mg, 7.93 mmol) of $SOCl_2$. The reaction mixture was allowed to warm slowly to room temperature and was then stirred for 6 h, diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 hexanes–ethyl acetate gave the expected product **2.64** as a yellowish solid: yield 832 mg (71%); silica gel TLC R_f 0.91 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.33 (s, 3H), 4.71 (s, 2H), 7.25 (d, 2H, J = 8.0 Hz), 7.48 (dd, 1H, J = 8.4 and 1.2 Hz), 7.69 (d, 1H, J = 8.4 Hz), 7.75 (s, 1H), 7.77 (s, 2H) and 8.26 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.6, 36.6, 108.3, 118.0, 118.8, 119.3, 120.9, 126.4, 127.0, 128.2, 130.4, 132.2, 134.2, 134.4 and 146.1; mass spectrum (APCI), *m*/*z* 345.0463 $(M+H)^+$ ($C_{17}H_{14}N_2O_2SCl$ requires *m*/*z* 345.0465).



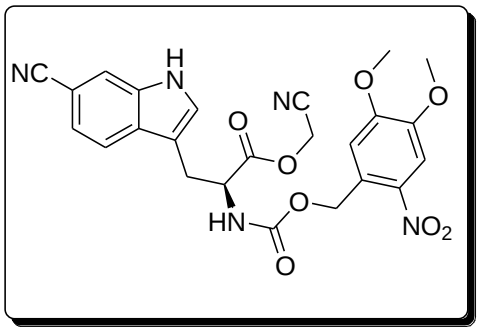
3-(((2S, 5R)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-indole-6-carbonitrile (2.65). To a stirred solution containing 0.62 mL (650 mg, 3.53 mmol) of Schöllkopf's reagent in 5 mL of anhydrous THF at −78 °C was added 0.45 mL (308 mg, 4.82 mmol) of 2.5 M BuLi. The reaction mixture was stirred at −78 °C for 30

min under argon and then a solution containing 1.10 g (3.21 mmol) of **2.64** in 5 mL of anhydrous THF was added. The reaction mixture was stirred at −78 °C for 30 min under argon, then diluted with 50 mL of satd aq NH_4Cl and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 hexanes–ethyl acetate gave the expected product **2.65** as a yellow oil: yield 1.01 g (65%); silica gel TLC R_f 0.89 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.57 (d, 3H, J = 6.8 Hz), 0.85 (d, 3H, J = 7.2 Hz), 2.03–2.09 (m, 1H), 2.34 (s, 3H), 3.12–3.19 (m, 3H), 3.61 (s, 3H), 3.63 (s, 3H), 4.27–4.30 (m, 1H), 7.23(d, 2H, J = 8.4 Hz), 7.41 (d, 1H, J = 8.0 Hz), 7.44 (s, 1H), 7.60 (d, 1H, J = 8.4 Hz), 7.67 (d, 2H, J = 8.0 Hz) and 8.23 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.6, 19.0, 21.7, 28.9, 31.6, 52.4, 52.5, 55.5, 60.6, 107.4, 117.9, 118.5, 119.7, 121.1, 125.9, 126.8, 128.1, 130.3, 133.8, 134.8, 134.9, 145.6, 161.9 and 164.3; mass spectrum (APCI), *m*/*z* 493.1910 $(M+H)^+$ ($C_{26}H_{29}N_4SO_4$ requires *m*/*z* 493.1909).



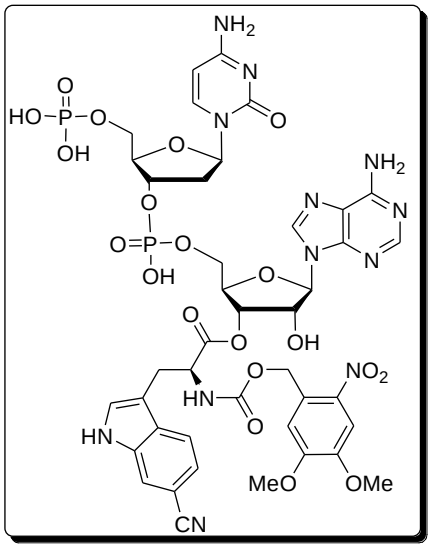
Methyl (S)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (2.67). To a stirred solution containing 468 mg (0.95 mmol) of **2.65** in 10 mL of THF at 0 °C was added 8 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then poured slowly

into 30 mL of satd aq $NaHCO_3$ and then extracted with two 30-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. Methyl (*S*)-2-amino-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.66**) was obtained as a yellow solid and was used directly in the next step without further purification. To a stirred solution containing 180 mg (0.45 mmol) of **2.66** in 2 mL of 1:1 dioxane–water was added 150 mg (1.08 mmol) of K_2CO_3 followed by 104 mg (0.48 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 14 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave the expected product **2.67** as a yellow oil: yield 256 mg (79% for two steps); silica gel TLC R_f 0.38 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.35 (s, 3H), 3.17–3.31 (m, 2H), 3.66 (s, 3H), 3.93 (s, 3H), 3.94 (s, 3H), 4.66–4.71 (m, 1H), 5.47–5.57 (m, 3H), 6.95 (s, 1H), 7.26 (d, 2H, J = 8.4 Hz), 7.44 (d, 1H, J = 8.0 Hz), 7.54 (s, 1H), 7.57 (s, 1H), 7.69–7.73 (m, 3H) and 8.24 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 27.7, 52.8, 54.0, 56.5, 56.6, 64.2, 108.0, 108.3, 110.4, 116.9, 118.1, 119.4, 120.4, 126.4, 126.89, 126.93, 127.4, 127.9, 130.3, 133.9, 134.0, 134.6, 146.0, 148.4, 153.7, 155.3 and 171.5; mass spectrum (APCI), *m*/*z* 637.1590 $(M+H)^+$ ($C_{30}H_{29}N_4O_{10}S$ requires *m*/*z* 637.1604).



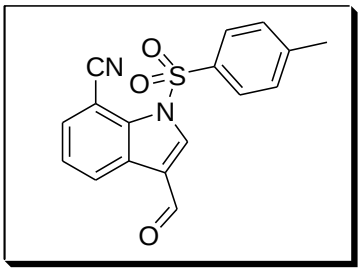
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (2.69). To a stirred solution containing 55.0 mg (0.08 mmol) **2.67** of in 1.5 mL of anhydrous 2:1 THF–methanol was added 56.5 mg (0.16 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 3 h under argon, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave **2.68** as a yellow oil: yield 27.0 mg (64%); silica gel TLC R_f 0.3 (1:1 hexanes–ethyl acetate). To a stirred solution containing 27.0 mg (0.05 mmol) of **2.68** in 1 mL of 1:3:1 water–THF–methanol was added 150 μL (3.59 mg, 0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was redissolved into 1 mL of anhydrous DMF and 24.7 μL (18.1 mg, 0.15 mmol) of Et_3N was added followed by 10.0 μL (11.3 mg, 0.15 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h and then diluted with 20 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:2 hexanes–ethyl acetate gave the desired

product **2.69** as a light yellow solid: yield 19.2 mg (44% for three steps); silica gel TLC R_f 0.25 (1:1 hexanes–ethyl acetate); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.12–3.27 (m, 2H), 3.84 (s, 3H), 3.87 (s, 3H), 4.43–4.48 (m, 1H), 5.00 (s, 2H), 5.33 (ABq, 2H, J = 14.8 Hz), 7.10 (s, 1H), 7.31 (d, 1H, J = 8.4 Hz), 7.52 (s, 1H), 7.69-7.73 (m, 2H), 7.85 (s, 1H), 8.22 (d, 1H, J = 7.6 Hz) and 11.50 (br s, 1H); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 26.1, 49.4, 54.5, 55.98, 56.03, 62.6, 102.3, 108.0, 110.2, 110.3, 115.5, 116.3, 119.2, 120.6, 121.1, 127.5, 128.6, 130.0, 134.8, 139.0, 147.6, 153.3, 155.5 and 170.9; mass spectrum (APCI), m/z 508.1488 $(M+H)^+$ ($C_{24}H_{22}N_5O_8$ requires m/z 508.1468).



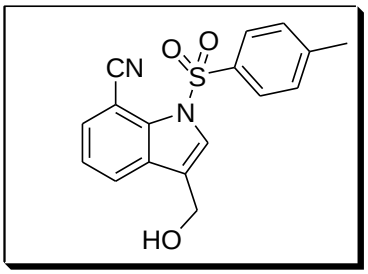
(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionic Acid pdCpA Ester (2.7). To a solution containing 5.20 mg (4.00 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 10.7 mg (21.0 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonyl)-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.69**). The reaction mixture was sonicated for 5 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM

aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 24.2 min. The fractions containing the product were lyophilized to afford **2.7** as a colorless solid: yield 2.90 mg (71%); mass spectrum (ESI), *m/z* 1085.2208 $(M-H)^-$ ($C_{41}H_{43}N_{12}O_{20}P_2$ requires *m/z* 1085.2192).



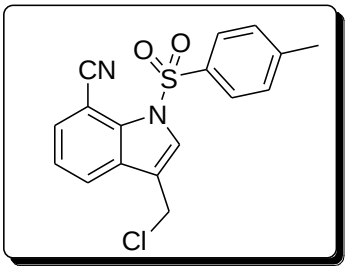
3-Formyl-1-tosyl-1*H*-indole-7-carbonitrile (2.71). Oxalyl chloride (0.6 mL, 0.89 g, 7.0 mmol) was added dropwise to 3 mL of cooled (ice-bath) DMF with stirring. The reaction mixture was then stirred at 0 °C for 1 h. A solution of 1.0 g (7.0 mmol) of 7-cyanoindole in DMF (3 mL) was then added to the reaction mixture in a dropwise manner. The resulting mixture was stirred at room temperature for overnight and 2 mL of 2 N NaOH was then added. The reaction mixture was heated at 100 ^oC for 30 min. The cooled reaction mixture was diluted with 50 mL of brine and then extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure to give 3-formyl-1*H*-indole-7-carbonitrile (**2.70**) as a yellow solid, which was used directly in the next step without further purification. To a stirred solution containing 954 mg (5.61 mmol) of **2.70** in 20 mL of anhydrous DMF at 0 °C was added 448 mg (11.2 mmol) of NaH (60% in mineral oil). The reaction mixture was stirred at 0 °C for 10 min under argon and then 1.49 g (7.85 mmol) of *p*TsCl was added. The reaction mixture was stirred at 0 °C under argon for 3 h. The reaction mixture was diluted with 150 mL of brine and extracted with two 50-mL portions of EtOAc. The organic

phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 4:1 hexanes–ethyl acetate gave the desired product **2.71** as a yellow solid: yield 1.50 g (66% for two steps); silica gel TLC R_f 0.78 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.36 (s, 3H), 7.31-7.39 (m, 3H), 7.67 (d, 1H, J = 7.6 Hz), 7.97 (d, 2H, J = 8.8 Hz), 8.53 (d, 1H, J = 8.4 Hz), 8.57 (s, 1H) and 10.13 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.6, 98.0. 116.7, 120.7, 124.9, 127.8, 128.3, 128.5, 130.3, 132.0, 133.69, 133.72, 139.2, 146.6 and 185.0; mass spectrum (APCI), m/z 325.0642 $(M+H)^+$ ($C_{17}H_{13}N_2SO_3$ requires m/z 325.0647).

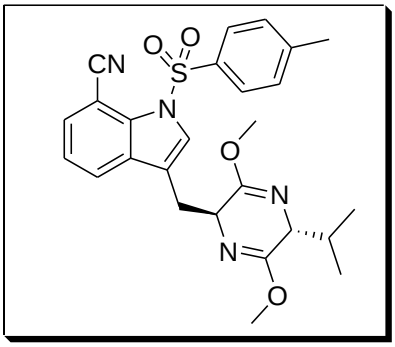


3-(Hydroxymethyl)-1-tosyl-1*H*-indole-7-carbonitrile (2.72). To a suspension of 880 mg (2.70 mmol) of 3-formyl-1-tosyl-1*H*-indole-7-carbonitrile (**2.71**) in 15 mL of EtOH was added 206 mg (5.42 mmol) of $NaBH_4$ and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with 100 mL of satd aq $NaHCO_3$ and the formed precipitate was filtered and dried under vacuum. 3-(Hydroxymethyl)-1-tosyl-1*H*-indole-7-carbonitrile (**2.72**) was obtained as an off-white solid: yield 682 mg (77%); silica gel TLC R_f 0.40 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.29 (s, 3H), 4.81 (s, 2H), 7.18-7.22 (m, 3H), 7.52 (d, 1H, J = 7.6 Hz), 7.78 (s, 1H) and 7.82-7.84 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.6, 56.4, 97.8, 117.4, 121.4, 123.1,

125.6, 126.9, 127.8, 130.1, 131.7, 132.0, 132.5, 134.8 and 145.7; mass spectrum (GCMS), *m*/*z* 326.0711 $(M)^+$ ($C_{17}H_{14}N_2SO_3$ requires *m*/*z* 326.0725).

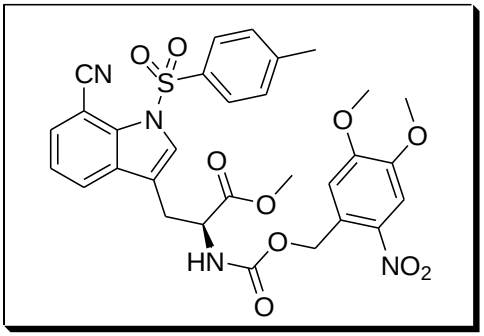


3-(Chloromethyl)-1-tosyl-1*H*-indole-7-carbonitrile (2.73). To a cooled (⁻10 °C) solution containing 831 mg (2.55 mmol) of 3-(hydroxymethyl)-1-tosyl-1*H*-indole-7-carbonitrile (**2.72**) in 15 mL of anhydrous CH_2Cl_2 and 1.42 mL (1.03 g, 10.2 mmol) of Et_3N was added dropwise 0.37 mL (606 mg, 5.09 mmol) of $SOCl_2$. The reaction mixture was allowed to warm slowly to room temperature and was then stirred for 6 h, diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 4:1 hexanes–ethyl acetate gave 3-(chloromethyl)-1-tosyl-1*H*-indole-7-carbonitrile (**2.73**) as a yellow oil: yield 650 mg (74%); silica gel TLC R_f 0.88 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.27 (s, 3H), 4.81 (s, 2H), 7.24 (d, 2H, *J* = 8.5 Hz), 7.29 (d, 1H, *J* = 5.0 Hz), 7.57 (d, 1H, *J* = 7.5 Hz), 7.69 (d, 1H, *J* = 8.0 Hz) and 7.92-7.95 (m, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.3, 36.5, 97.8, 116.9, 117.7, 123.0, 125.0, 127.6, 128.0, 129.9, 130.8, 131.6, 132.5, 134.3 and 145.6; mass spectrum (APCI), *m*/*z* 345.0466 $(M+H)^+$ ($C_{17}H_{14}N_2O_2SCl$ requires *m*/*z* 345.0465).



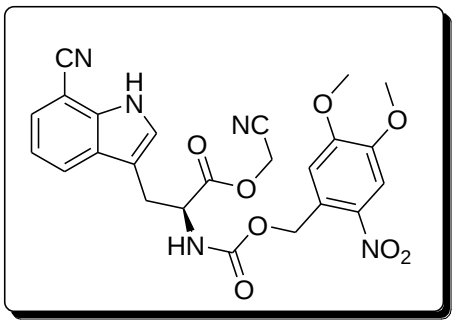
3-(((2*S*, 5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-indole-7-carbonitrile (2.74). To a stirred solution containing 0.35 mL (365 mg, 1.98 mmol) of Schöllkopf's reagent in 5 mL of anhydrous THF at ⁻78 °C was added 0.25 mL (172 mg, 2.68 mmol) of 2.5 M BuLi. The reaction mixture was stirred at ⁻78 °C for 30 min under argon, and then a solution containing 618 mg (1.79 mmol) of 3-(chloromethyl)-1-tosyl-1*H*-indole-7-carbonitrile (**2.73**) in 5 mL of anhydrous THF was added. The reaction mixture was stirred at ⁻78 °C for 30 min under argon, then diluted with 50 mL of satd aq NH_4Cl and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 hexanes–ethyl acetate gave 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-indole-7-carbonitrile (**2.74**) as a yellow oil: yield 547 mg (62%); silica gel TLC R_f 0.85 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.58 (d, 3H, J = 6.8 Hz), 0.88 (d, 3H, J = 6.8 Hz), 2.07-2.12 (m, 1H), 2.32 (s, 3H), 3.19-3.32 (m, 3H), 3.63 (s, 6H), 4.30-4.31 (m, 1H), 7.18-7.24 (m, 3H), 7.53 (d, 1H, J = 7.6 Hz), 7.55 (s, 1H) and 7.79-7.81 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.5, 19.0, 21.6, 28.7, 31.6, 52.3, 52.4, 55.6, 60.6, 97.9, 117.1, 117.4, 122.5,

125.4, 127.7, 127.8, 130.0, 131.4, 132.2, 133.7, 135.2, 145.3, 162.0 and 164.1; mass spectrum (APCI), *m*/*z* 493.1909 $(M+H)^+$ ($C_{26}H_{29}N_4SO_4$ requires *m*/*z* 493.1909).



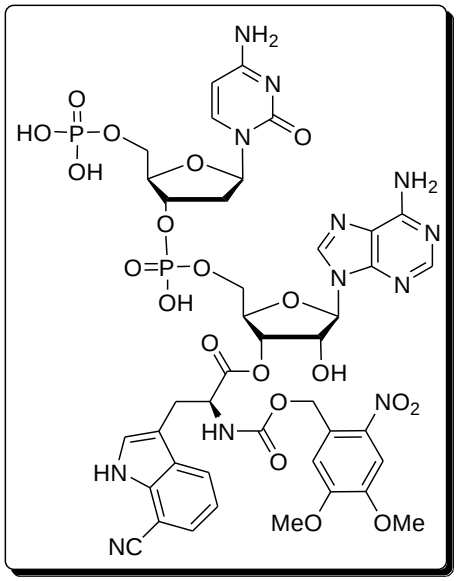
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (2.76). To a stirred solution containing 466 mg (0.94 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-indole-7-carbonitrile (**2.74**) in 16 mL of THF at 0 °C was added 14 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 3 h. The reaction mixture was then slowly poured into 50 mL of satd aq $NaHCO_3$ and then extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. Methyl (*S*)-2-amino-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.75**) was obtained as a yellow oil and was used directly in the next step without further purification. To a stirred solution containing 179 mg (0.44 mmol) of **2.75** in 2 mL of 1:1 dioxane–water was added 149 mg (1.07 mmol) of K_2CO_3 followed by 104 mg (0.48 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 12 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-

dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.76**) as a yellow oil: yield 237 mg (73% for two steps); silica gel TLC R_f 0.35 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.30 (s, 3H), 3.16-3.32 (m, 2H), 3.66 (s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 4.66-4.68 (m, 1H), 5.42 (ABq, 2H, J = 15.2 Hz), 5.78 (d, 1H, J = 7.6 Hz), 6.92 (s, 1H), 7.21-7.24 (m, 3H), 7.54 (d, 1H, J = 7.6 Hz), 7.61 (s, 1H), 7.67 (s, 1H), 7.73 (d, 1H, J = 7.6 Hz) and 7.80 (d, 2H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.6, 27.4, 52.7, 54.0, 56.3, 56.4, 63.9, 98.2, 108.1, 110.0, 115.9, 117.1, 123.1, 124.6, 127.6, 127.7, 130.0, 130.1, 131.7, 132.5, 132.8, 134.8, 139.5, 145.6, 148.1, 153.7, 155.3 and 171.6; mass spectrum (APCI), *m*/*z* 637.1578 $(M+H)^+$ ($C_{30}H_{29}N_4O_{10}S$ requires *m*/*z* 637.1604).

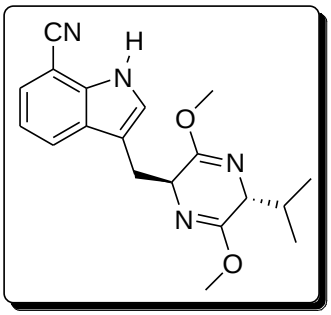


Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (2.78). To a stirred solution containing 160 mg (0.25 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.76**) in 3 mL of 2:1 anhydrous THF–methanol was added 261 mg (0.74 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 3 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on

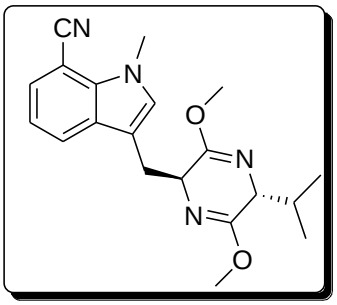
a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.77**) as a yellow oil. To a stirred solution containing 43.0 mg (0.09 mmol) of **2.77** in 1 mL of 1:3:1 water–THF–methanol was added 275 µL (6.59 mg, 0.27 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was dissolved in 1 mL of anhydrous DMF under argon. To the stirred solution was added 38.0 µL (28.0 mg, 0.27 mmol) of Et_3N followed by 17.0 µL (20.0 mg, 0.27 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h and then diluted with 20 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with 1:2 hexanes–ethyl acetate gave cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.78**) as a light yellow solid: yield 23.0 mg (55% for three steps); silica gel TLC R_f 0.21 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.34-3.37 (m, 2H), 3.92 (s, 3H), 3.93 (s, 3H), 4.62-4.68 (m, 1H), 4.80 (d, 2H, J = 15.2 Hz), 5.45-5.51 (m, 2H), 6.92 (s, 1H), 7.17-7.21 (m, 2H), 7.51 (d, 1H, J = 7.6 Hz), 7.67 (s, 1H), 7.80 (d, 1H, J = 8.0 Hz) and 9.08 (br s, 1H); mass spectrum (APCI), m/z 508.1470 $(M+H)^+$ ($C_{24}H_{22}N_5O_8$ requires m/z 508.1468).



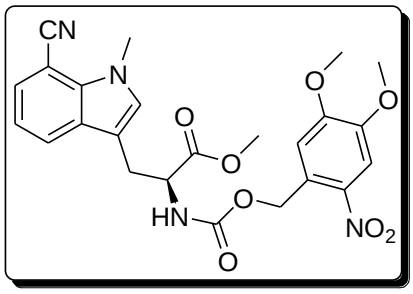
(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionic Acid pdCpA Ester (2.8). To a solution containing 5.24 mg (3.85 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 10.7 mg (21.1 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.78**). The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 21.6 min. The fractions containing the product were lyophilized to afford **2.8** as a colorless solid: yield 2.70 mg (65%); mass spectrum (ESI), *m/z* 1085.2209 $(M-H)^-$ ($C_{41}H_{43}N_{12}O_{20}P_2$ requires *m/z* 1085.2192).



3-(((2*S*, 5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1*H*-indole-7-carbonitrile (2.79). To a stirred solution containing 326 mg (0.67 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-tosyl-1*H*-indole-7-carbonitrile (**2.74**) in 5 mL of 2:1 anhydrous THF–methanol was added 699 mg (1.98 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for overnight under argon. The reaction mixture was poured into 20 mL of brine, and then extracted with two 20-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1*H*-indole-7-carbonitrile (**2.79**) as a yellow oil: yield 152 mg (68%); silica gel TLC R_f 0.75 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.62 (d, 3H, J = 6.8 Hz), 0.93 (d, 3H, J = 7.2 Hz), 2.13-2.15 (m, 1H), 3.29 (d, 2H, J = 8.8 Hz), 3.41 (d, 1H, J = 3.6 Hz), 3.64 (s, 3H), 3.67 (s, 3H), 4.33-4.36 (m, 1H), 7.05-7.12 (m, 2H), 7.46 (d, 1H, J = 7.6 Hz), 7.88 (d, 1H, J = 8.0 Hz) and 9.00 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.6, 19.1, 29.4, 31.6, 52.3, 56.5, 60.6, 94.2, 113.0, 117.7, 118.9, 124.6, 125.0, 126.4, 128.2, 129.2, 136.3, 162.7 and 164.1; mass spectrum (APCI), *m*/*z* 339.1817 $(M+H)^+$ ($C_{19}H_{23}N_4O_2$ requires *m*/*z* 339.1821).

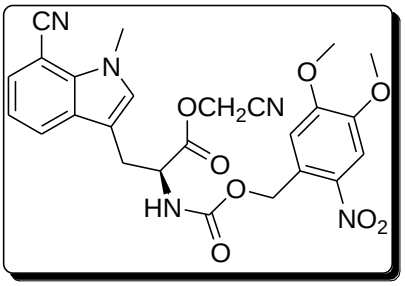


3-(((2*S*, 5*R*)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-methyl-1*H*-indole-7-carbonitrile (2.80). To a stirred solution containing 156 mg (0.46 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1*H*-indole-7-carbonitrile (**2.79**) in 3 mL of anhydrous DMF at 0 °C was added 21.4 mg (0.89 mmol) of NaH followed by 0.06 mL (127 mg, 0.89 mmol) of MeI. The reaction mixture was stirred at 0 °C for 1 h under argon. The reaction mixture was diluted with 100 mL of satd aq $NaHCO_3$ and was extracted with two 20-mL portions of EtOAc and then dried under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-methyl-1*H*-indole-7-carbonitrile (**2.80**) as a yellow oil: yield 120 mg (74%); silica gel TLC R_f 0.78 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.61 (d, 3H, J = 6.8 Hz), 0.93 (d, 3H, J = 6.8 Hz), 2.10-2.15 (m, 1H), 3.14-3.20 (m, 1H), 3.50-3.55 (m, 1H), 3.59-3.60 (m, 1H), 3.61 (s, 3H), 3.66 (s, 3H), 3.68 (s, 3H), 4.31-4.33 (m, 1H), 6.79 (s, 1H), 7.01-7.06 (m, 1H), 7.44 (d, 1H, J = 7.6 Hz) and 7.83 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.6, 19.1, 29.1, 31.7, 34.6, 52.3, 52.4, 56.6, 60.7, 93.4, 111.5, 118.3, 119.0, 125.1, 128.2, 130.3, 130.6, 134.8, 162.8 and 164.0; mass spectrum (APCI), *m*/*z* 353.1971 $(M+H)^+$ ($C_{20}H_{25}N_4O_2$ requires *m*/*z* 353.1977).



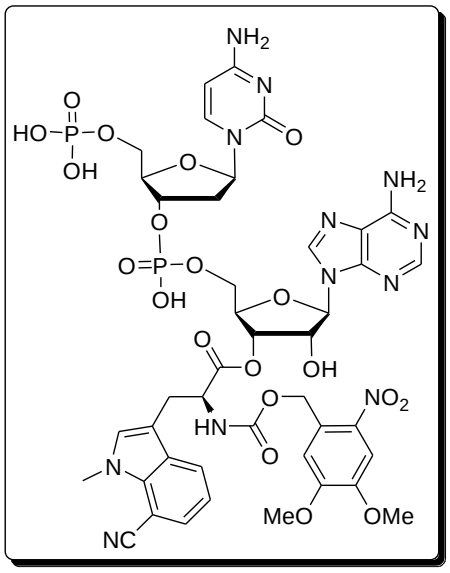
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (2.82). To a stirred solution containing 117 mg (0.33 mmol) of 3-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1-methyl-1*H*-indole-7-carbonitrile (**2.80**) in 4 mL of THF at 0 °C was added 4 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then poured slowly into 50 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. Methyl (*S*)-2-amino-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (**2.81**) was obtained as a yellow oil and was used directly in the next step without further purification. To a stirred solution containing 31.0 mg (0.12 mmol) of **2.81** in 2 mL of 1:1 dioxane–water was added 56.0 mg (0.41 mmol) of K_2CO_3 followed by 39.0 mg (0.18 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 12 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (**2.82**) as a yellow oil: yield 33.1 mg (65% for two steps); silica gel TLC R_f 0.25 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.27-3.31 (m, 2H), 3.69

(s, 3H), 3.90 (s, 3H), 3.93 (s, 3H), 4.07 (s, 3H), 4.66-4.71 (m, 1H), 5.47-5.49 (m, 2H), 6.92 (s, 2H), 7.07-7.10 (m, 1H), 7.50 (d, 1H, J = 7.6 Hz), 7.68 (s, 1H) and 7.71 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.4, 34.7, 52.6, 54.5, 56.46, 56.50, 64.0, 94.0, 108.2, 109.5, 110.3, 118.6, 119.0, 124.1, 127.7, 128.7, 129.7, 130.3, 135.0, 139.8, 148.2, 153.6, 155.3 and 172.1; mass spectrum (APCI), m/z 497.1677 $(M+H)^+$ ($C_{24}H_{25}N_4O_8$ requires m/z 497.1672).



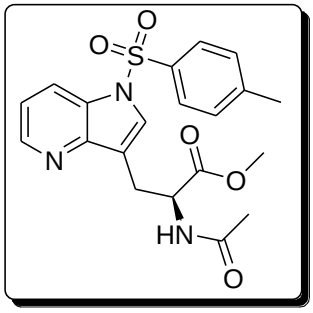
Cyanomethyl (*S*)- 2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (2.83). To a stirred solution containing 35.0 mg (0.07 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (**2.82**) in 1 mL of 1:3:1 water–THF–methanol was added 214 μL (5.13 mg, 0.21 mmol) of 1 N aq LiOH. The reaction mixture was stirred at room temperature for 3 h, then concentrated under diminished pressure. The residue was dissolved in 1 mL of anhydrous DMF and 30.0 μL (22.0 mg, 0.21 mmol) of Et_3N was added followed by 13.0 μL (16.0 mg, 0.21 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h and then diluted with 20 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with 1:1 hexanes–ethyl acetate gave cyanomethyl (*S*)-2-((4,5-dimethoxy-2-

nitrobenzyloxy)carbonylamino)-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (**2.83**) as a light yellow solid: yield 20.0 mg (52%); silica gel TLC R_f 0.42 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.87-2.89 (m, 1H), 3.34-3.35 (m, 1H), 3.93 (s, 3H), 3.96 (s, 3H), 4.10 (s, 3H), 4.62-4.69 (m, 1H), 4.75-4.85 (m, 2H), 5.37 (d, 1H, J = 8.0 Hz), 5.51 (ABq, 2H, J = 14.0 Hz), 6.92 (s, 1H), 7.00 (s, 1H), 7.13-7.16 (m, 1H), 7.56 (d, 1H, J = 7.5 Hz), 7.70 (s, 1H) and 7.75 (d, 1H, J = 8.0 Hz); mass spectrum (APCI), m/z 522.1623 $(M+H)^+$ ($C_{25}H_{24}N_5O_8$ requires m/z 522.1625).



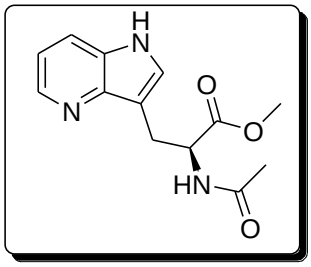
(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionic Acid pdCpA Ester (2.9). To a solution containing 5.24 mg (3.85 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 11.0 mg (21.1 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy) carbonylamino)-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (**2.83**). The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention

time of the desired product was 22.0 min. The fractions containing the product were lyophilized to afford **2.9** as a colorless solid: yield 2.90 mg (69%); mass spectrum (ESI), *m/z* 1099.2358 $(M-H)^-$ ($C_{42}H_{45}N_{12}O_{20}P_2$ requires *m/z* 1099.2348).

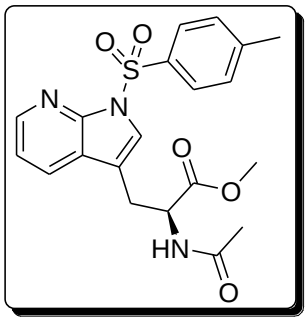


Methyl (*S*)-2-Acetamido-3-(1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (2.84). To a stirred solution containing 16.0 mg (0.04 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.16**) in 3 mL dry CH_2Cl_2 under a N_2 atmosphere was added 13.0 μL (10.3 mg, 0.08 mmol) of DIPEA followed by 4.0 μL (4.10 mg, 0.04 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then diluted with 20 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave methyl (*S*)-2-acetamido-3-(1-tosyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.84**) as a yellow oil: yield 15.1 mg (79%); silica gel TLC R_f 0.25 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.95 (s, 3H), 2.32 (s, 3H), 3.17-3.30 (m, 2H), 3.56 (s, 3H), 4.73-4.76 (m, 1H), 7.21-7.25 (m, 2H), 7.58 (s, 1H), 7.69 (d, 2H, *J* = 8.4 Hz), 8.23 (dd, 1H, *J* = 8.4 and 1.6 Hz), 8.31 (d, 1H, *J* = 6.8 Hz) and 8.48 (dd, 1H, *J* = 4.8 and 1.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 23.2, 27.0, 52.2, 52.4, 118.8, 119.7, 121.5, 126.8,

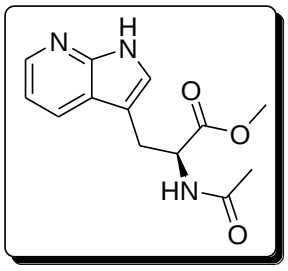
128.1, 129.1, 130.3, 134.9, 145.6, 145.8, 147.9, 170.3 and 171.6; mass spectrum (APCI), *m*/*z* 416.1265 $(M+H)^+$ ($C_{20}H_{22}N_3O_5S$ requires *m*/*z* 416.1280).



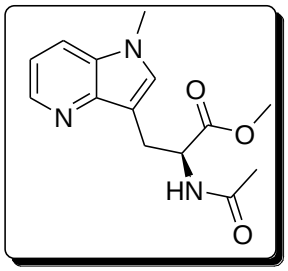
Methyl (*S*)-2-Acetamido-3-(1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (2.85). To a stirred solution containing 12.0 mg (29 μmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.84**) in 1 mL of 2:1 THF–methanol was added 31.1 mg (87 μmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 1 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with ethyl acetate gave the expected product methyl (*S*)-2-acetamido-3-(1*H*-pyrrolo[3,2-b]pyridin-3-yl)propionate (**2.85**) as a yellow solid: yield 4.10 mg (58%); silica gel TLC R_f 0.45 (10:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.03 (s, 3H), 3.27-3.40 (m, 2H), 3.62 (s, 3H), 4.71-4.76 (m, 1H), 7.12-7.15 (m, 1H), 7.23-7.24 (m, 1H), 7.66 (d, 1H, *J* = 8.0 Hz), 8.43 (d, 1H, *J* = 4.8 Hz), 8.65 (s, 1H) and 9.13-9.14 (m, 1H); mass spectrum (APCI), *m*/*z* 262.1187 $(M+H)^+$ ($C_{13}H_{16}N_3O_3$ requires *m*/*z* 262.1192).



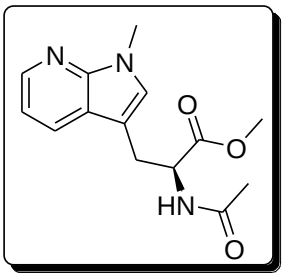
Methyl (*S*)-2-Acetamido-3-(1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (2.86). To a stirred solution containing 32.0 mg (0.08 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo [2,3-*b*]pyridin-3-yl)propionate (**2.25**) in 3 mL dry CH_2Cl_2 under a N_2 atmosphere was added 26 μL (20.7 mg, 0.16 mmol) of DIPEA followed by 8.0 μL (8.16 mg, 0.08 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then diluted with 50 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave methyl (*S*)-2-acetamido-3-(1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.86**) as a yellow oil: yield 31.0 mg (79%); silica gel TLC R_f 0.25 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.93 (s, 3H), 2.32 (s, 3H), 3.11-3.25 (m, 1H), 3.65 (s, 3H), 4.83-4.88 (m, 1H), 6.35 (d, 1H, J = 7.6 Hz), 7.12-7.15 (m, 1H), 7.22 (d, 2H, J = 8.4 Hz), 7.48 (s, 1H), 7.78 (d, 1H, J = 7.6 Hz), 7.98 (d, 1H, J = 8.0 Hz) and 8.37 (dd, 1H, J = 4.8 and 1.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.6, 23.1, 27.7, 52.5, 52.6, 113.8, 118.8, 123.1, 124.4, 127.88, 127.94, 129.7, 135.3, 145.2, 145.3, 147.3, 170.0 and 171.9; mass spectrum (APCI), *m*/*z* 416.1293 $(M+H)^+$ ($C_{20}H_{22}N_3O_5S$ requires *m*/*z* 416.1280).



Methyl (*S*)-2-Acetamido-3-(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (2.87). To a stirred solution containing 25.0 mg (60 μmol) of methyl (*S*)-2-acetamido-3-(1-tosyl-1H-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.86**) in 1 mL of 2:1 anhydrous THF–methanol was added 43.1 mg (120 μmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 1 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with ethyl acetate gave the expected product methyl (*S*)-2-acetamido-3-(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.87**) as a yellow solid: yield 11.1 mg (69 %); silica gel TLC R_f 0.45 (10:1 ethyl acetate-methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.01 (s, 3H), 3.25-3.38 (m, 2H), 3.60 (s, 3H), 4.69-4.74 (m, 1H), 7.10-7.13 (m, 1H), 7.22-7.23 (m, 1H), 7.65 (d, 1H, J = 8.0 Hz), 8.42 (d, 1H, J = 4.8 Hz), 8.64 (s, 1H) and 9.12-9.13 (m, 1H); mass spectrum (APCI), m/z 262.1192 $(M+H)^+$ ($C_{13}H_{16}N_3O_3$ requires m/z 262.1192).

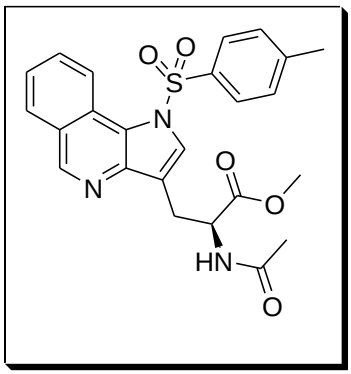


Methyl (*S*)-2-Acetamido-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (2.88). To a stirred solution containing 9.10 mg (0.04 mmol) of methyl (*S*)-2-amino-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.31**) in 3 mL dry CH_2Cl_2 under a N_2 atmosphere was added 13.0 μL (10.3 mg, 0.08 mmol) of DIPEA followed by 4.0 μL (4.10 mg, 0.04 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 2 h, then diluted with 20 mL of satd aq $NaHCO_3$ and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with ethyl acetate gave methyl (*S*)-2-acetamido-3-(1-methyl-1*H*-pyrrolo[3,2-*b*]pyridin-3-yl)propionate (**2.88**) as a yellow oil: yield 8.10 mg (81%); silica gel TLC R_f 0.25 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.01 (s, 3H), 3.23-3.37 (m, 2H), 3.64 (s, 3H), 3.75 (s, 3H), 4.69-4.73 (m, 1H), 7.10 (s, 1H), 7.13-7.16 (m, 1H), 7.60 (dd, 1H, J = 8.0 and 1.2 Hz), 8.41 (d, 1H, J = 4.4 Hz) and 9.03-9.05 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.2, 27.2, 33.0, 52.1, 54.6, 110.7, 116.9, 117.1, 130.4, 131.3, 142.3, 145.6, 170.7 and 172.2; mass spectrum (APCI), *m*/*z* 276.1341 $(M+H)^+$ ($C_{14}H_{18}N_3O_3$ requires *m*/*z* 276.1348).



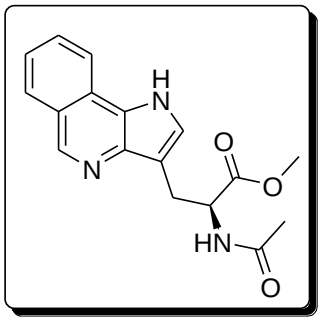
Methyl (*S*)-2-Acetamido-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (2.89). To a stirred solution containing 80.0 mg (0.15 mmol) of methyl (*S*)-2-amino-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.36**) in 3 mL dry CH_2Cl_2 under a N_2

atmosphere was added 50.0 μL (38.8 mg, 0.30 mmol) of DIPEA followed by 15.0 μL (15.2 mg, 0.15 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then diluted with 50 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave methyl (*S*)-2-acetamido-3-(1-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)propionate (**2.89**) as a yellow oil: yield 29.1 mg (67 %); silica gel TLC R_f 0.1 (1:2 hexanes-ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.93 (s, 3H), 3.19-3.25 (m, 2H), 3.64 (s, 3H), 3.79 (s, 3H), 4.84-4.88 (m, 1H), 6.92 (s, 1H), 6.98-7.01 (m, 1H), 7.77 (d, 1H, J = 7.6 Hz) and 8.26 (d, 1H, J = 4.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.2, 27.7, 31.0, 52.4, 57.2, 107.2, 115.3, 120.5, 126.9, 127.5, 143.1, 147.8, 169.9 and 172.4; mass spectrum (APCI), *m*/*z* 276.1349 $(M+H)^+$ ($C_{14}H_{18}N_3O_3$ requires *m*/*z* 276.1348).



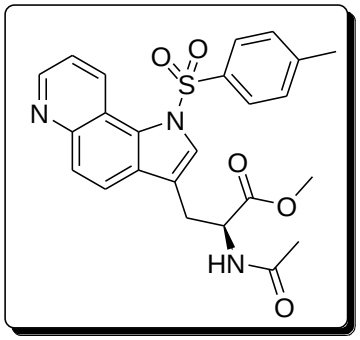
Methyl (*S*)-2-Acetamido-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (2.90). To a stirred solution containing 16.0 mg (0.04 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.48**) in 3 mL of dry DCM under N_2 atmosphere was added 0.02 mL (10.3 mg, 0.08 mmol) of DIPEA followed by 4.0 μL (4.1

mg, 0.04 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 1 h, diluted with 30 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with hexanes–ethyl acetate 1:1 gave the expected product methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.90**) as a yellow oil: yield 14.0 mg (79%); silica gel TLC R_f 0.45 (1:1 hexanes–ethyl acetate); ^{1}HNMR ($CDCl_3$, 400 MHz) δ 1.99 (s, 3H), 2.27 (s, 3H), 3.39-3.40 (m, 2H), 3.69 (s, 3H), 4.82-4.84 (m, 1H), 7.15 (d, 2H, J = 8.0 Hz), 7.52-7.59 (m, 3H), 7.75-7.79 (m, 1H), 7.86 (s, 1H), 8.03 (d, 1H, J = 8.0 Hz), 8.31-8.33 (m, 1H) and 9.05-9.07 (m, 2H), ^{13}CNMR ($CDCl_3$, 100 MHz) δ 21.7, 23.2, 26.9, 52.3, 53.7,118.7, 123.0, 123.6, 125.8, 126.2, 126.8, 126.9, 128.7, 129.3, 130.2, 131.5, 135.1, 143.2, 145.6, 150.6, 170.4 and 171.8; mass spectrum (APCI) *m*/*z* 466.1439 $(M+H)^+$ ($C_{24}H_{24}N_3O_5S$ requires *m*/*z* 466.1437).



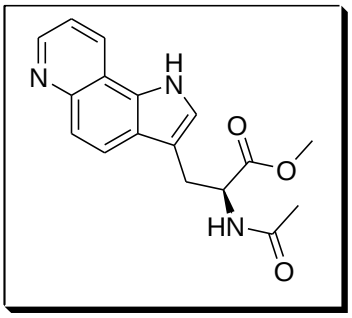
Methyl (*S*)-2-Acetamido-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (2.91). To a stirred solution containing 14.0 mg (0.03 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.90**) in 1 mL of 2:1 anhydrous THF–methanol was added 32.1 mg (0.09 mmol) of Cs_2CO_3. The reaction mixture was stirred at

room temperature for 3 h under argon, diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution ethyl acetate gave the expected product methyl (*S*)-2-acetamido-3-(1*H*-pyrrolo[3,2-*c*]isoquinolin-3-yl)propionate (**2.91**) as a yellow solid: yield 5.10 mg (57%); silica gel TLC R_f 0.45 (10:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.10 (s, 3H), 3.40-3.42 (m, 2H), 3.66 (s, 3H), 4.78-4.80 (m, 1H), 7.10-7.11 (m, 1H), 7.47-7.50 (m, 1H), 7.66-7.67 (m, 1H), 7.94-7.97 (m, 2H), 8.91 (d, 1H, J = 7.5 Hz), 9.16-9.20 (m, 1H) and 9.45 (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 23.3, 27.4, 52.2, 54.9, 113.1, 119.4, 122.9, 123.4, 124.6, 125.0, 125.2, 129.0, 130.3, 130.3, 146.2, 170.9 and 172.6; mass spectrum (APCI) *m*/*z* 312.1354 $(M+H)^+$ ($C_{17}H_{18}N_3O_3$ requires *m*/*z* 312.1348).



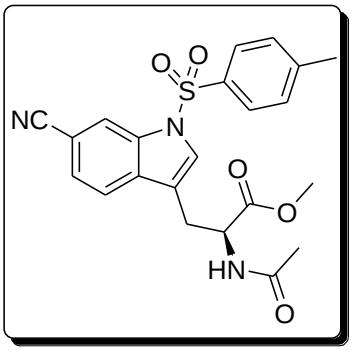
Methyl (*S*)-2-Acetamido-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (2.92). To a stirred solution containing 80.0 mg (0.15 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.57**) in 3 mL of dry DCM under N_2 atmosphere was added 0.05 mL (38.8 mg, 0.30 mmol) of DIPEA followed by 15.0 µL (15.2 mg, 0.15 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was then diluted with 50 mL of satd aq

$NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave the expected product methyl (*S*)-2-acetamido-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.92**) as a yellow oil: yield 75.1 mg (85%); silica gel TLC R_f 0.25 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.98 (s, 3H), 2.27 (s, 3H), 3.28-3.32 (m, 1H), 3.40-3.44 (m, 1H), 3.72 (s, 3H), 4.96-4.99 (m, 1H), 6.13 (d, 1H, *J* = 7.5 Hz), 7.11 (d, 2H, *J* = 8.0 Hz), 7.41-7.43 (m, 1H), 7.45 (d, 2H, *J* = 8.5 Hz), 7.71 (s, 1H), 7.76 (d, 1H, *J* = 9.0 Hz), 7.93 (d, 1H, *J* = 8.5 Hz), 8.81-8.83 (m, 1H) and 9.44 (d, 1H, *J* = 8.5 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.7, 23.4, 27.3, 52.8, 52.9, 117.5, 119.0, 121.0, 121.3, 126.8, 127.3, 128.6, 129.8, 129.9, 130.1, 132.2, 134.9, 145.5, 147.4, 148.7, 169.9 a nd 171.9; mass spectrum (APCI) *m*/*z* 466.1447 $(M+H)^+$ ($C_{24}H_{24}N_3O_5S$ requires *m*/*z* 466.1437).



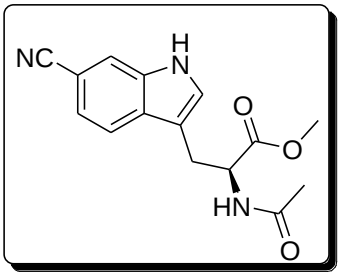
Methyl (*S*)-2-Acetamido-3-(1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (2.93). To a stirred solution containing 42.0 mg (0.09 mmol) of methyl (*S*)-2-amino-3-(1-tosyl-1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.92**) in 2 mL of 2:1 anhydrous THF–methanol was added 96.0 mg (0.27 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 2 h under argon, diluted with 50 mL of brine and extracted with two 50-

mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with hexanes–ethyl acetate 1:1 gave the expected product methyl (*S*)-2-acetamido-3-(1*H*-pyrrolo[2,3-*f*]quinolin-3-yl)propionate (**2.93**) as a yellow oil: yield 17.1 mg (59%); silica gel TLC R_f 0.2 (10:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 500 MHz) δ 1.93 (s, 3H), 3.24-3.29 (m, 1H), 3.36-3.41 (m, 1H), 3.68 (s, 3H), 4.77-4.80 (m, 1H), 7.28 (s, 1H), 7.53-7.56 (m, 1H), 7.65 (d, 1H, J = 8.5 Hz), 7.95 (d, 1H, J = 9.0 Hz) and 8.71 (d, 2H, J = 8.0 Hz); ^{13}C NMR (CD_3OD, 125 MHz) δ 22.4, 28.4, 52.8, 55.3, 113.4, 120.4, 121.5, 124.1, 124.4, 124.9, 130.9, 131.3, 146.5, 146.5, 147.7, 17 3.2 and 173.9; mass spectrum (APCI) *m*/*z* 312.1339 $(M+H)^+$ ($C_{17}H_{18}N_3O_3$ requires *m*/*z* 312.1348).



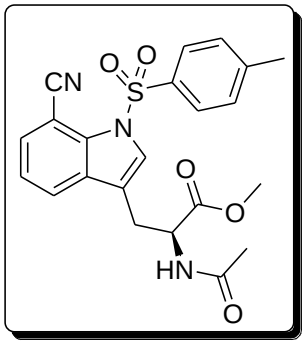
Methyl (*S*)-2-Acetamido-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (2.94). To a stirred solution containing 20.0 mg (0.05 mmol) of methyl (*S*)-2-amino-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.66**) in 3 mL of dry CH_2Cl_2 under a N_2 atmosphere was added 17.4 μL (12.9 mg, 0.10 mmol) of DIPEA followed by 5.0 μL (5.1 mg, 50 μmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 2 h, then diluted with 20 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished

pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-acetamido-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.94**) as a yellow oil: yield 17.2 mg (78%); silica gel TLC R_f 0.45 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.95 (s, 3H), 2.35 (s, 3H), 3.15-3.29 (m, 2H), 3.66 (s, 3H), 4.86-4.88 (m, 1H), 6.27 (br s, 3H), 7.46-7.57 (m, 3H), 7.71-7.73 (m, 2H) and 8.23 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.7, 23.2, 27.5, 52.4, 52.7, 107.9, 117.2, 118.0, 119.4, 120.5, 126.3, 126.9, 127.8, 130.3, 133.9, 134.0, 134.6, 145.9, 169.9 and 171.7; mass spectrum (APCI), *m*/*z* 440.1272 $(M+H)^+$ ($C_{22}H_{22}N_3O_5S$ requires *m*/*z* 440.1280).



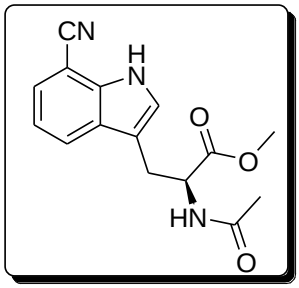
Methyl (*S*)-2-Acetamido-3-(6-cyano-1*H*-indol-3-yl)propionate (2.95). To a stirred solution containing 13.0 mg (29.0 μmol) of methyl (*S*)-2-acetamido-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.94**) in 1 mL of 2:1 anhydrous THF–methanol was added 31.2 mg (87.1 μmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 1 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with ethyl acetate gave methyl (*S*)-2-acetamido-3-(6-cyano-1*H*-indol-3-yl)propionate (**2.95**) as a yellow solid: yield 5.10 mg (57%); silica gel TLC R_f 0.50 (ethyl acetate); ^{1}H NMR (CD_3OD, 400 MHz) δ 1.91 (s, 3H), 3.15-3.20 (m, 2H), 3.66

(s, 3H), 4.71-4.74 (m, 1H), 7.30 (d, 1H, *J* = 8.4 Hz), 7.37 (s, 1H), 7.67 (d, 1H, *J* = 8.4 Hz) and 7.75 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 22.4, 28.2, 52.8, 54.9, 104.5, 112.2, 117.4, 120.4, 121.7, 122.6, 129.1, 132.1, 136.8, 173.2 and 173.7; mass spectrum (APCI), *m*/*z* 286.1199 $(M+H)^+$ ($C_{15}H_{16}N_3O_3$ requires *m*/*z* 286.1192).

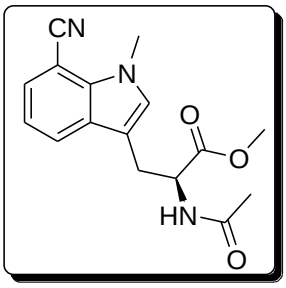


Methyl (*S*)-2-Acetamido-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (2.96). To a stirred solution containing 32.0 mg (0.08 mmol) of methyl (*S*)-2-amino-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.75**) in 3 mL of dry CH_2Cl_2 under a N_2 atmosphere was added 26.8 μL (20.7 mg, 0.16 mmol) of DIPEA followed by 7.56 μL (8.16 mg, 0.08 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then diluted with 50 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with 1:1 hexanes–ethyl acetate gave methyl (*S*)-2-acetamido-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.96**) as a yellow oil: yield 28.1 mg (80%); silica gel TLC R_f 0.41 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.93 (s, 3H), 2.32 (s, 3H), 3.11-3.25 (m, 2H), 3.65 (s, 3H), 4.83-4.88 (m, 1H), 6.35 (d, 1H, *J* = 7.6 Hz), 7.12-7.15 (m, 1H), 7.22 (d, 2H, *J* = 8.4 Hz), 7.48 (s, 1H), 7.78

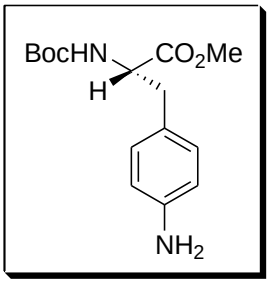
(d, 1H, *J* = 7.6 Hz), 7.98 (d, 1H, *J* = 8.0 Hz) and 8.37 (dd, 1H, *J* = 4.8 and 1.2 Hz); mass spectrum (APCI), *m*/*z* 440.1278 $(M+H)^+$ ($C_{22}H_{22}N_3O_5S$ requires *m*/*z* 440.1280).



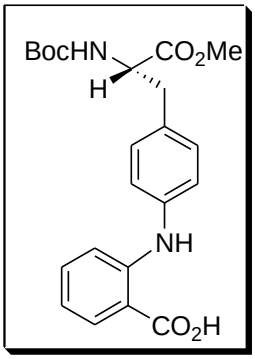
Methyl (*S*)-2-Acetamido-3-(7-cyano-1*H*-indol-3-yl)propionate (2.97). To a stirred solution containing 26.2 mg (0.06 mmol) of methyl (*S*)-2-acetamido-3-(7-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**2.96**) in 1 mL of 2:1 anhydrous THF–methanol was added 43.1 mg (120 μmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 1 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with ethyl acetate gave methyl (*S*)-2-acetamido-3-(7-cyano-1*H*-indol-3-yl)propionate (**2.97**) as a yellow solid: yield 12.0 mg (68%); silica gel TLC R_f 0.18 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.01 (s, 3H), 3.25-3.38 (m, 2H), 3.60 (s, 3H), 4.69-4.74 (m, 1H), 7.10-7.13 (m, 1H), 7.22-7.23 (m, 1H), 7.65 (d, 1H, *J* = 8.0 Hz), 8.42 (d, 1H, *J* = 4.8 Hz), 8.64 (s, 1H) and 9.12-9.13 (m, 1H); mass spectrum (APCI), *m*/*z* 286.1192 $(M+H)^+$ ($C_{15}H_{16}N_3O_3$ requires *m*/*z* 286.1192).



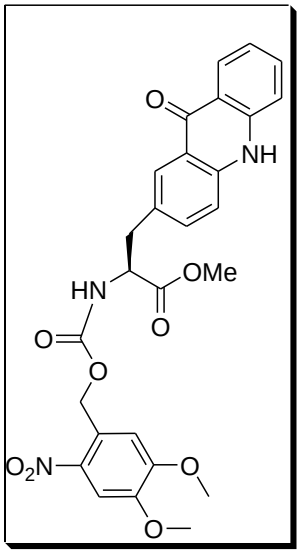
Methyl (*S*)-2-Acetamido-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (2.98). To a stirred solution containing 38.6 mg (0.15 mmol) of methyl (*S*)-2-amino-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (**2.81**) in 3 mL dry CH_2Cl_2 under a N_2 atmosphere was added 50.8 μL (38.8 mg, 0.30 mmol) of DIPEA followed by 15.0 μL (15.2 mg, 0.15 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 2 h, diluted with 50 mL of satd aq $NaHCO_3$ and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with ethyl acetate gave methyl (*S*)-2-acetamido-3-(7-cyano-1-methyl-1*H*-indol-3-yl)propionate (**2.98**) as a yellow oil: yield 30.0 mg (69%); silica gel TLC R_f 0.21 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.93 (s, 3H), 3.20-3.26 (m, 2H), 3.65 (s, 3H), 4.02 (s, 3H), 4.84-4.88 (m, 1H), 6.17 (s, 1H), 6.84 (s, 1H), 7.07 (t, 1H, J = 8.0 Hz), 7.46 (d, 1H, J = 7.2 Hz) and 7.69 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.2, 27.2, 34.6, 52.5, 53.0, 93.9, 109.8, 118.6, 119.0, 124.2, 128.6, 129.9, 130.1, 134.9, 169.8 and 172.4; mass spectrum (APCI), *m*/*z* 300.1344 $(M+H)^+$ ($C_{16}H_{18}N_3O_3$ requires *m*/*z* 300.1348).



Methyl (*S*)-3-(4-Aminophenyl)-2-(*tert*-butoxycarbonylamino)propionate (2.99). To a cooled (⁻10 °C) solution containing 1.00 g (3.50 mmol) of (*S*)-3-(4-aminophenyl)-2-(*tert*-butoxycarbonylamino)propionic acid in 10 mL of anhydrous MeOH was added dropwise 0.25 mL (416 mg, 3.50 mmol) of $SOCl_2$. The reaction mixture was left to warm slowly to room temperature and stirred for 3 h, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 4:1 hexanes–ethyl acetate gave **2.99** as a yellow oil: yield 594 mg (55%); silica gel TLC R_f 0.7 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.34 (s, 9H), 2.87 (t, 2H, J = 6.4 Hz), 3.59 (s, 3H), 3.68 (s, 2H), 4.40-4.42 (m, 1H), 5.14 (d, 1H, J = 8.0 Hz), 6.49 (d, 2H, J = 8.4 Hz) and 6.80 (d, 2H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.4, 37.1, 51.8, 54.5, 79.4, 114.9, 125.0, 129.8, 145.5, 155.0 and 172.4; mass spectrum (APCI), *m*/*z* 295.1652 $(M+H)^+$ ($C_{15}H_{23}N_2O_4$ requires *m*/*z* 295.1658).

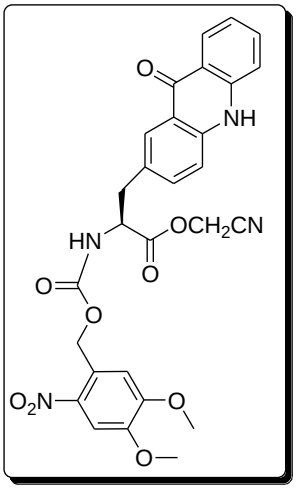


(*S*)-2-(4-(2-(*tert*-Butoxycarbonylamino)-3-methoxy-3-oxopropyl)phenylamino) benzoic acid (2.100). A mixture containing 3.32 g (11.3 mmol) of **2.99**, 0.88 g (5.65 mmol) of 2-chlorobenzoic acid, 0.39 g (2.8 mmol) of K_2CO_3, 0.23 g of Cu powder in 4 mL DMF was heated at reflux for 2 h. After cooling to room temperature, the reaction mixture was filtered, the filtrate slowly added to 30 mL of H_2O, and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 4:1 hexanes–ethyl acetate gave **2.100** as a yellow oil: yield 1.10 g (47%); silica gel TLC R_f 0.7 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.44 (s, 9H), 3.01-3.15 (m, 2H), 3.74 (s, 3H), 4.61-4.63 (m, 1H), 5.12-5.14 (m, 1H), 6.73-6.76 (m, 1H), 7.11-7.20 (m, 4H), 7.31-7.35 (m, 1H), 8.04 (d, 1H, J = 8.0 Hz) and 9.34 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.4, 38.0, 52.4, 54.6, 80.2, 110.9, 114.1, 117.3, 123.1, 130.4, 131.6, 132.7, 135.1, 139.5, 148.8, 155.3, 172.6 and 173.3; mass spectrum (APCI), *m*/*z* 415.1871 $(M+H)^+$ ($C_{22}H_{27}N_2O_6$ requires *m*/*z* 415.1869).



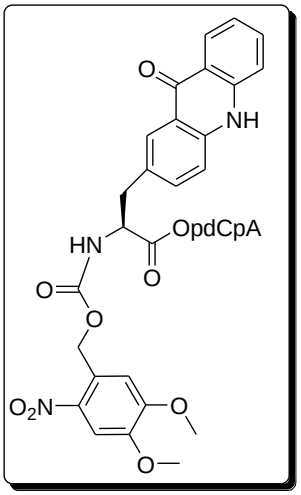
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(9-oxo-9,10-dihydroacridin-2-yl)propionate (2.102). Polyphosphoric acid (13.0 mL) was preheated to 80 °C and added to 413 mg (1.35 mmol) of **2.101**. The reaction mixture was stirred mechanically at 100 °C for 1 h, cooled to 50 °C, and poured into 50 mL of ice-water and and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was redissolved 4 mL of dioxane-water (1:1) and 83.1 mg (4.22 mmol) of K_2CO_3 was added to the reaction mixture followed by 336 mg (1.56 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 14 h under argon. The reaction mixture was then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexanes–ethyl acetate gave the expected product **2.102** as a yellow oil: yield 51.0 mg (10% for two steps); silica gel TLC R_f 0.55 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.16-3.35 (m, 2H), 3.79 (s, 3H), 3.89 (s, 3H), 3.92 (s, 3H), 4.71-4.72 (m, 1H), 5.47-5.52

(m, 2H), 6.94 (s, 1H), 7.27-7.31 (m, 3H), 7.64-7.67 (m, 2H), 8.24 (s, 1H), 8.29 (br s, 1H) and 8.44 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 36.0, 51.9, 55.5, 56.0, 62.4, 108.0, 109.9, 117.2, 117.3, 120.2, 120.3, 120.8, 125.9, 126.0, 127.8, 129.9, 133.3, 134.4, 138.9, 139.6, 140.7, 147.5, 153.3, 155.5, 172.1 and 176.5; mass spectrum (APCI), m/z 536.1671 $(M+H)^+$ ($C_{27}H_{26}N_3O_9$ requires m/z 536.1669).



Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(9-oxo-9,10-dihydroacridin-2-yl)propionate (2.103). To a stirred solution containing 46.0 mg (0.09 mmol) of methyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(9-oxo-9,10-dihydroacridin-2-yl)propionate (**2.102**) in 1 mL of 1:3:1 water–THF–methanol was added 275 µL (6.46 mg, 0.27 mmol) of 1N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was redissolved in 1 mL of anhydrous DMF under argon. To the stirred solution was added 38.0 µL (28.0 mg, 0.27 mmol) of Et_3N followed by 17.0 µL (20.0 mg, 0.27 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h and then diluted with 20 mL of EtOAc. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by

chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave the expected product cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(9-oxo-9,10-dihydroacridin-2-yl)propionate (**2.103**) as a light yellow solid: yield 20.0 mg (40%); silica gel TLC R_f 0.5 ethyl acetate; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.00-3.22 (m, 2H), 3.77 (s, 3H), 3.81(s, 3H), 4.41-4.47 (m, 3H), 5.69 (ABq, 2H, J = 14.8 Hz), 7.04 (s, 1H), 7.19-7.23 (m, 1H), 7.43 (d, 1H, J = 8.4 Hz), 7.49 (d, 1H, J = 8.4 Hz), 7.62-7.71 (m, 3H), 8.11 (s, 2H), 8.18 (d, 1H, J = 8.0 Hz) and 8.25 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 35.6, 49.5, 55.2, 56.0, 62.5, 108.0, 110.0, 115.5, 117.2, 117.4, 120.2, 120.4, 120.8, 125.9, 126.2, 127.5, 129.5, 133.3, 134.4, 139.0, 139.7, 140.7, 147.6, 153.3, 155.5, 170.8 and 176.5; mass spectrum (APCI), *m*/*z* 561.1613 $(M+H)^+$ ($C_{28}H_{25}N_4O_9$ requires *m*/*z* 561.1621).



(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(9-oxo-9,10-dihydroacridin-2-yl)-pdCpA (2.10). To a solution containing 5.20 mg (4.0 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 12.1 mg (21.0 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(9-oxo-9,10-dihydroacridin-2-yl)propionate (**2.103**).

The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a reversed phase column (C_{18}, 10 × 250 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate (pH 4.5)–acetonitrile. The retention time of the desired product was 23.4 min. The fractions containing the product were lyophilized to afford **2.103** as a colorless solid: yield 1.51 mg (34%); mass spectrum (ESI), *m*/*z* 1138.2372 $(M-H)^-$ ($C_{45}H_{46}N_{11}O_{21}P_2$ requires *m*/*z* 1138.2345.

CHAPTER 3

SYNTHESIS OF ALANYL NUCLEOBASE AMINO ACIDS FOR INCORPORATION INTO DNA-BINDING PROTEINS

3.1. Introduction

Molecules which can bind with high sequence specificity to a chosen target in a gene sequence are of interest for the development of gene therapeutic agents, diagnostic devices for genetic analysis, and as molecular tools for nucleic acid manipulations.[149] Since interactions between nucleobases are important for controlling nucleic acid structures and gene expression, and base-base recognition can be highly selective, polypeptides containing nucleobases at defined positions could potentially be used to bind to DNA and RNA specifically.[150,151]

Peptide nucleic acid (PNA), first described by Nielsen's group in 1991, is a DNA analogue in which the entire negatively charged sugar-phosphate backbone has been replaced with a neutral, peptide-like backbone usually formed from *N*-2-aminoethylglycine units (Figure 3.1).[152] PNA is achiral and uncharged. It is chemically stable and not prone to degradation by nucleases or proteases, and thus not expected to be degraded inside a living cell.[149] Owing to its neutral backbone and proper interbase spacing, a PNA molecule binds to its complementary nucleic acid sequence (both single-stranded DNA and RNA as well as double-stranded DNA) with higher affinity compared to natural DNA and RNA.[149,152] The neutral backbone also predicts a lack of electrostatic repulsion between the PNA and DNA strands (compared to that existing between two negatively-charged DNA oligomers), and hence a higher thermal stability of PNA–DNA duplexes.[152] Although the poor cellular uptake of free PNA is still considered a major

obstacle for the application of PNA for gene targeting, PNA has attracted major attention due to its potential for diagnostic[153,154] as well as biotechnological[155,156] and pharmaceutical[157,158] applications.

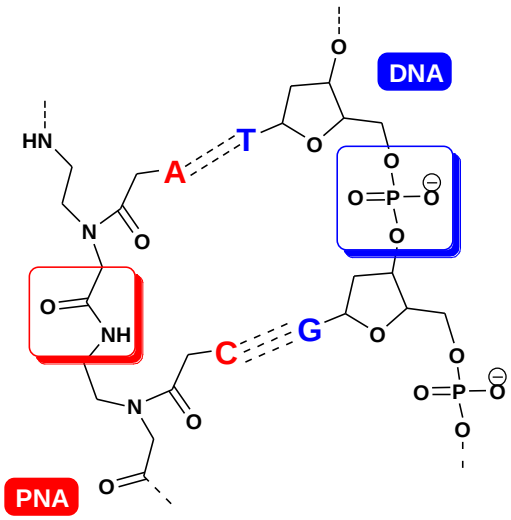


Figure 3.1. Schematic chemical model of PNA and DNA, showing their different backbone linkages.

Although PNA contains an amide bond in its backbone, it has never been observed to form protein-like tertiary structures. Therefore, a simple PNA molecule may not discriminate highly structured nucleic acids in the same fashion as nucleic acid binding proteins. To obtain molecules capable of recognizing nucleobases in nucleic acids, but also capable of forming protein-like structues, a series of peptides containing L-α-amino acids with a nucleobase side chain (nucleobase amino acid (NBA)) was designed by Mihara's group.[159-161] They reported the use of L-amino-γ-nucleobase-butyric acids (nucleobase amino acids or NBAs) (Figure 3.2) for the construction of different RNA binding peptides. In order to realize selective binding within more complex protein–DNA structures, our laboratory has employed the same strategy involving nucleobase interaction for DNA–protein interaction, anticipating that the

nucleobase moieties in the proteins could specifically recognize base sequence in nucleic acids through H-bonding and base stacking interactions.

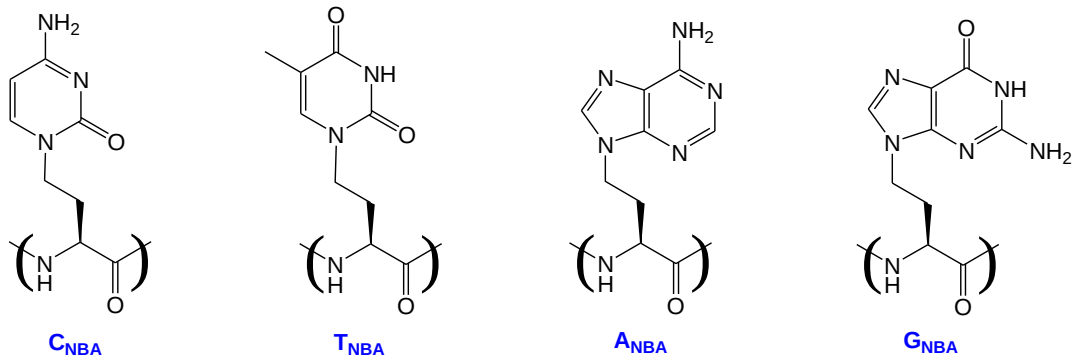


Figure 3.2. Chemical structures of the cytosine (C_{NBA}), thymine (T_{NBA}), adenine (A_{NBA}), and guanine (G_{NBA}) residues within the RNA binding peptide chain.

Recently, Diederichsen's group presented an enantioselective preparation of alanyl nucleobase amino acids with MMT-protected α-amino groups and acyl-protected exocyclic amino functions of the nucleobases for the solid phase synthesis of alanyl-PNA/DNA chimeras as a tool for very sensitive DNA detection (Figure 3.3).[162] In this thesis, a series of alanyl nucleobase amino acids (**ANA-1** – **ANA-8**) (Figure 3.4) have been prepared. A site-specific mutagenesis technique, which permits the insertion of unnatural amino acids into any predetermined position of a protein by the suppression of TAG or four base codons with misacylated-tRNAs, has not been used before for incorporation of amino acids having nucleobase side chains. Therefore, this strategy was attempted for the incorporation of **ANA**s into the DNA-binding protein RRM1. In order to synthesize the requisite aminoacylated tRNAs for protein synthesis, the pdCpA derivatives (**3.1**-**3.8**) of alanyl nucleobase amino acids were prepared (Figure 3.5).

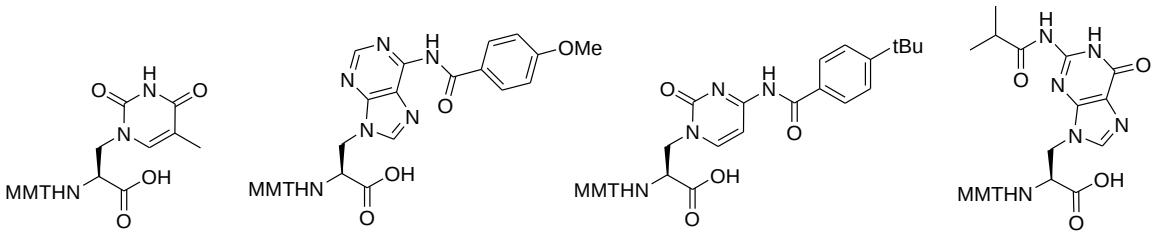


Figure 3.3. Chemical structures of the MMT/acyl-protected nucleobase amino acids for the solid phase synthesis of DNA/alanyl-PNA chimeras.[162]

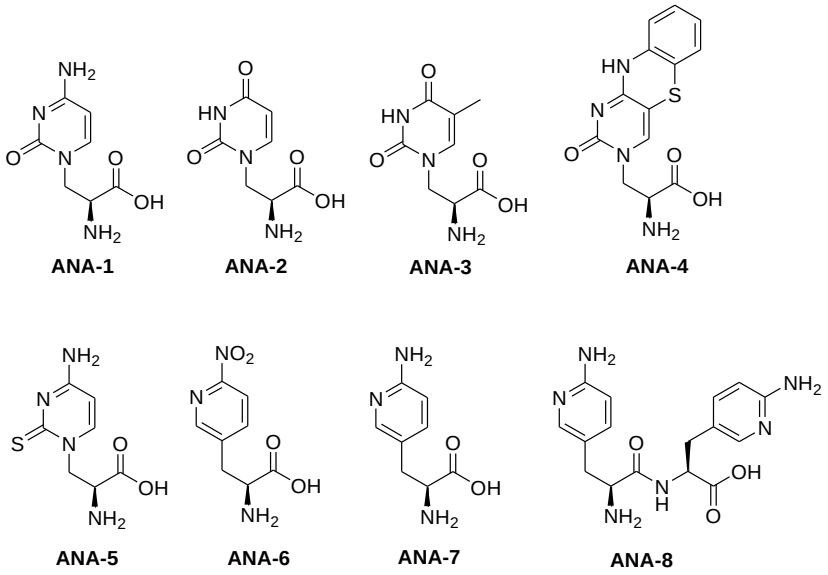


Figure 3.4. Series of alanyl nucleobase amino acids synthesized for site-directed incorporation at position 24 of RRM1.

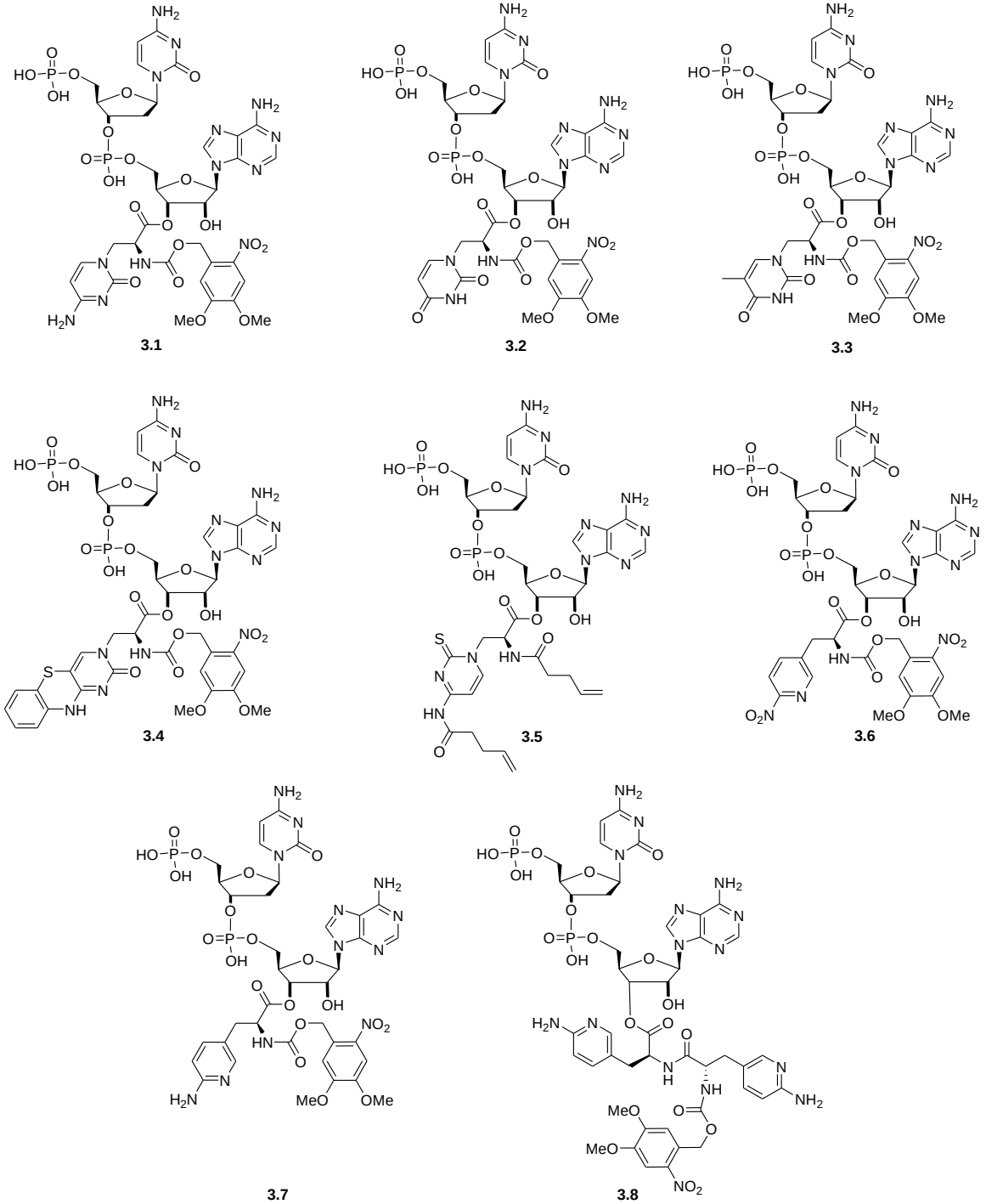
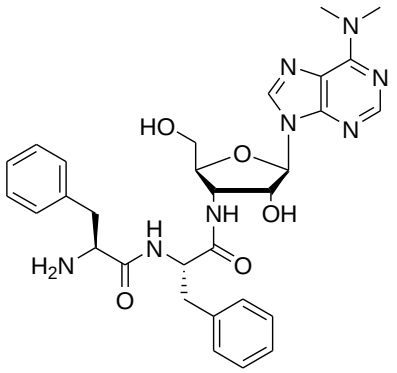


Figure 3.5. Series of aminoacylated pdCpA derivatives synthesized for site-directed incorporation at position 24 of RRM1.

As discussed Chapter 1, ribosomes having modifications in their 23S rRNA enabled the incorporation of the dipeptides and dipeptidomimetics into DHFR have been realized. The selection of ribosomes having modifications in specific regions of the 23S rRNA was done using puromycin derivatives. Using the same strategy, a dipeptidylpuromycin **3.9** (Figure 3.6) was synthesized to enable the selection of ribosomes that could accept the dipeptide **ANA-8** during protein synthesis.



3.9

Figure 3.6. Structure of puromycin derivative **3.9**.

The protein RRM1 (14 kD) is a DNA binding domain of human transcription factor hnRNP LL.[163] The transcription factor hnRNP LL (heterogeneous nuclear ribonucleoprotein) has been shown to bind to the i-motif in the *BCL2* promoter region, and to unfold the i-motif to initiate transcription. hnRNP LL has four RNA recognition motif (RRM) domains, each potentially capable of binding to the i-motif.

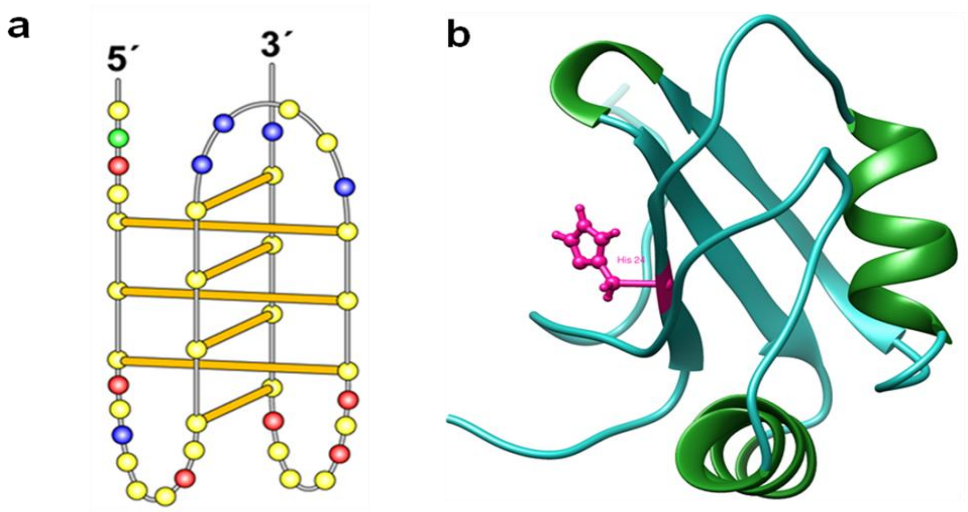


Figure 3.7. (a) Structure of i-motif DNA.[163] The cytidine rich i-motif stem is shown in yellow with two of the lateral loops and the central loop: blue–T; red –G ; green –A. **(b)** Structure of RRM1 of human hnRNP LL, including His24 generated by I-Tasser software using the structure of Mus musculus RRM domain of BAB28521 protein (pdb 1WEX) as a template.[164]

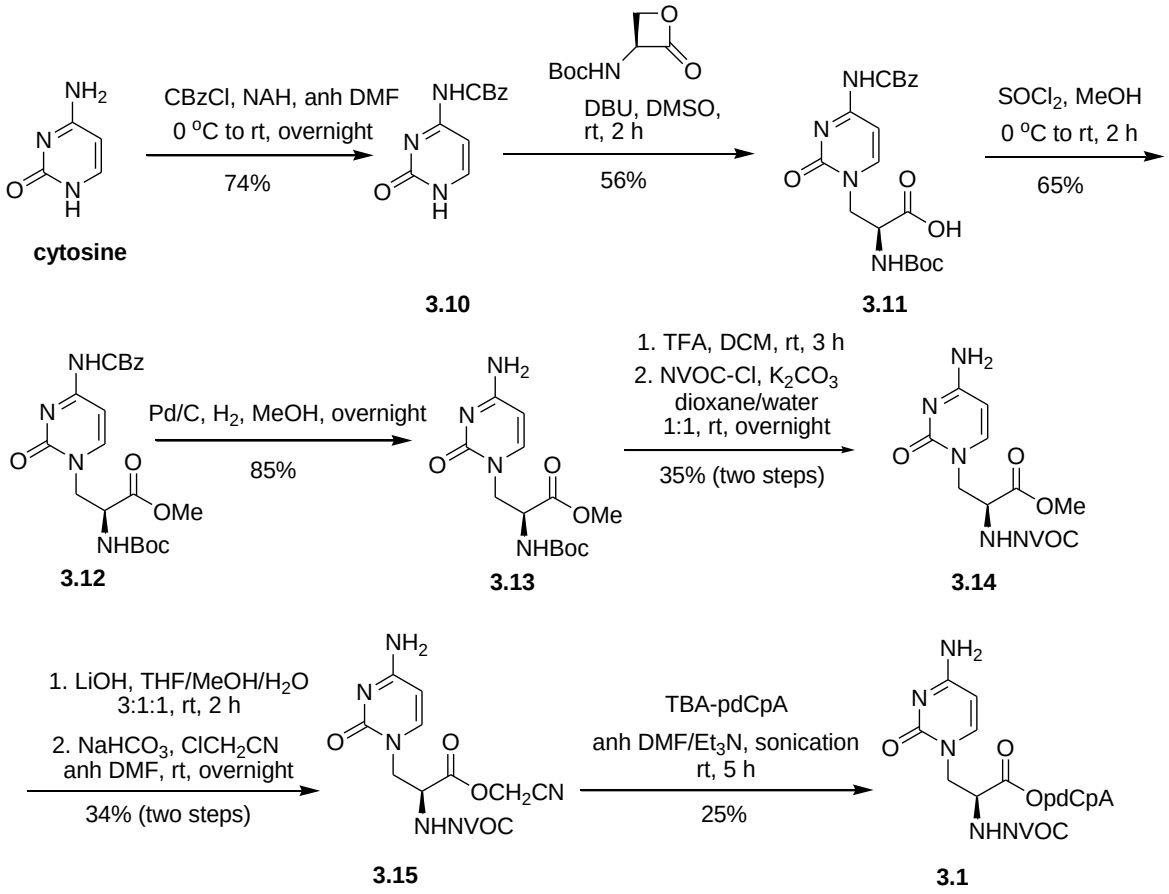
3.2. Results

Synthesis of pdCpAs Esters

In this study, proteins have been synthesized with different **ANA** units as constituents to enable a study of the ability of the protein nucleobase moiety to enforce the selectivity of nucleic acid interaction. In order to synthesize the requisite aminoacylated tRNAs for protein synthesis, the pdCpA derivatives were prepared from their respective amino acids. The asymmetric syntheses of alanyl nucleobase amino amino acids were acheived either by nucleophilic ring opening of *N*-Boc-L-serine β-lactone, or by using the Schöllkopf chiral auxiliary.

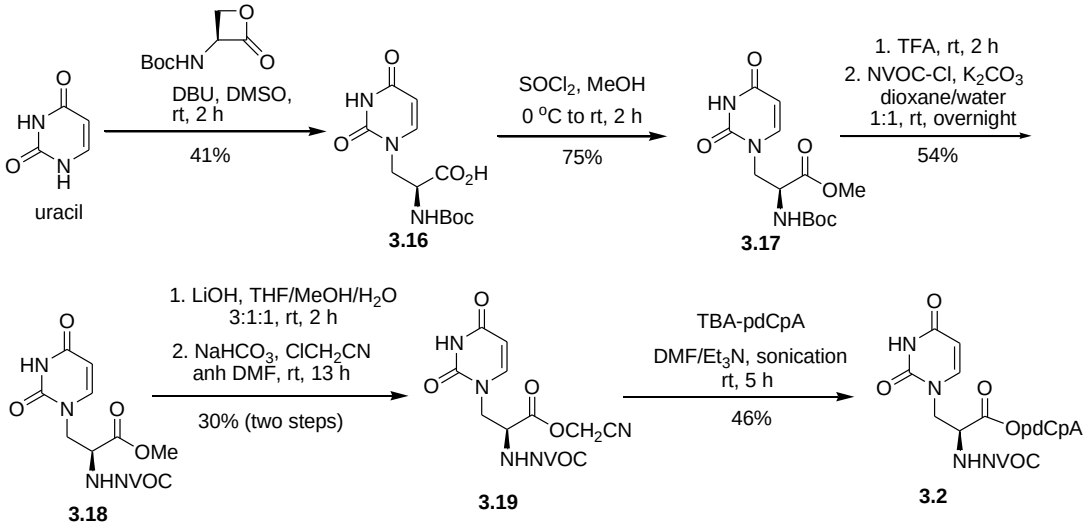
The synthesis of the aminoacylated pdCpA derivative of alanyl nucleobase amino acid **ANA-1** was accomplished starting from commercially available cytosine (Scheme 3.1). CBz protection of cytosine afforded compound **3.10** in 74% yield.[165] Compound

3.10 underwent coupling with *N*-Boc-L-serine β-lactone to afford compound **3.11** in 56% yield,[162] the latter of which reacted with $SOCl_2$ in methanol to form methyl ester **3.12**. The CBz protecting group was removed by hydrogenolysis over Pd/C to afford **3.13**. Boc deprotection and subsequent NVOC protection of compound **3.13** afforded compound **3.14** in 35% overall yield. *N*-protected methyl ester **3.14** was subsequently hydrolyzed to afford the free acid, the latter of which was treated with chloroacetonitrile to afford the desired cyanomethyl ester **3.15** in 34% yield over two steps. The key intermediate cyanomethyl ester of the *N*-protected amino acid (**3.15**) was coupled with tris(tetrabutylammonium) salt of pdCpA to give a pdCpA ester **3.1** in 25% yield.



Scheme 3.1. Synthesis of **ANA-1** and its Aminoacyl-pdCpA.

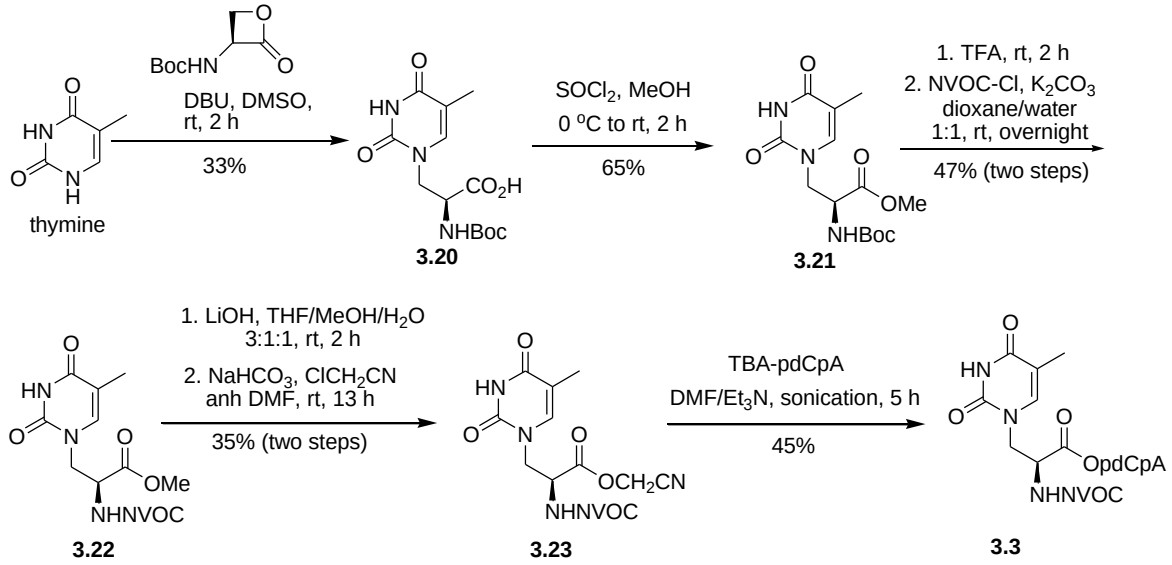
The synthesis of the aminoacylated pdCpA derivative of alanyl nucleobase amino acid **ANA-2** was accomplished starting from commercially available uracil (Scheme 3.2) which underwent coupling with *N*-Boc-L-serine β-lactone to afford compound **3.16** in 41% yield. Free acid **3.16** reacted with $SOCl_2$ in methanol to form the methyl ester **3.17** in 75% yield. Boc deprotection and subsequent NVOC protection of methyl ester **3.17** afforded NVOC-carbamate **3.18** in 54% yield. Methyl ester **3.18** was subjected to saponification in the presence of aqueous LiOH to produce the free acid, which was activated as cyanomethyl ester **3.19** in 30% overall yield. Treatment of cyanomethyl ester **3.19** with the tris(tetrabutylammonium) salt of pdCpA in anhydrous DMF afforded pdCpA ester **3.2** in 46% yield.



Scheme 3.2. Synthesis of **ANA-2** and its Aminoacyl-pdCpA.

The synthesis of the aminoacylated pdCpA derivative of amino acid **ANA-3** was accomplished starting from commercially available thymine (Scheme 3.3) which underwent coupling with *N*-Boc-L-serine β-lactone to afford compound **3.20** in 33% yield. Compound **3.20** was treated with $SOCl_2$ in methanol to afford the methyl ester **3.21**

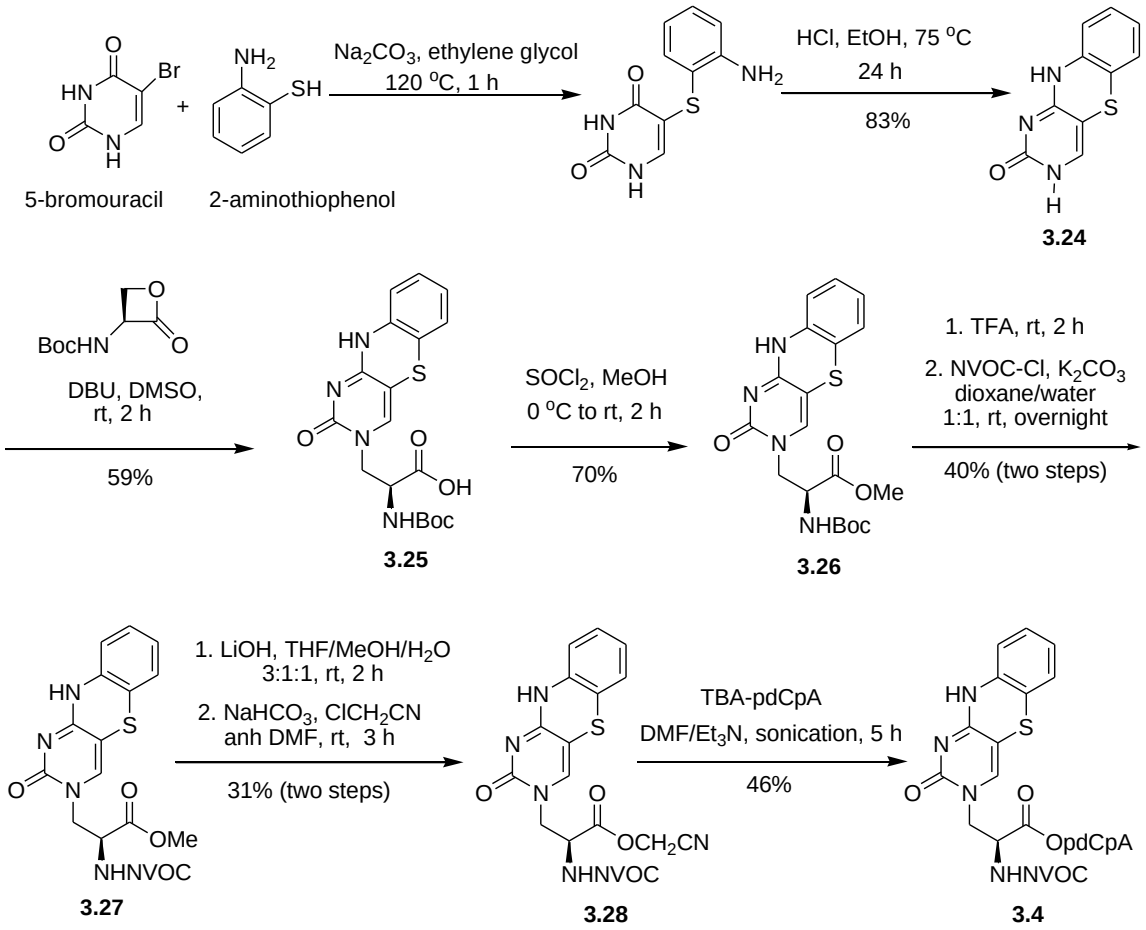
in 65% yield. Boc deprotection and subsequent NVOC protection of methyl ester **3.21** afforded NVOC-carbamate **3.22** in 47% overall yield. NVOC methyl ester **3.22** was hydrolyzed to afford the free acid, the latter of which was treated with chloroacetonitrile to afford the desired cyanomethyl ester **3.23** in 35% yield over two steps. Cyanomethyl ester **3.23** was used to ligate the dinucleotide pdCpA to afford the pdCpA ester **3.3** in 45% yield.



Scheme 3.3. Synthesis of **ANA-3** and its Aminoacyl-pdCpA.

The synthesis of the aminoacylated pdCpA derivative of amino acid **ANA-4** was accomplished starting from 1,3-diaza-2-oxophenothiazine[166,167] which was synthesized from commercially available 5-bromouracil and 2-aminothiophenol following a literature procedure[167] (Scheme 3.4). Compound **3.24** underwent coupling with *N*-Boc-L-serine β-lactone to afford compound **3.25** in 59% yield. Free acid **3.25** was treated with $SOCl_2$ in methanol to form the methyl ester **3.26** in 70% yield. Boc deprotection and subsequent NVOC protection of methyl ester **3.26** afforded NVOC-protected methyl ester **3.27** in 40% yield over two steps. Hydrolysis of NVOC methyl ester **3.27** afforded the free acid,

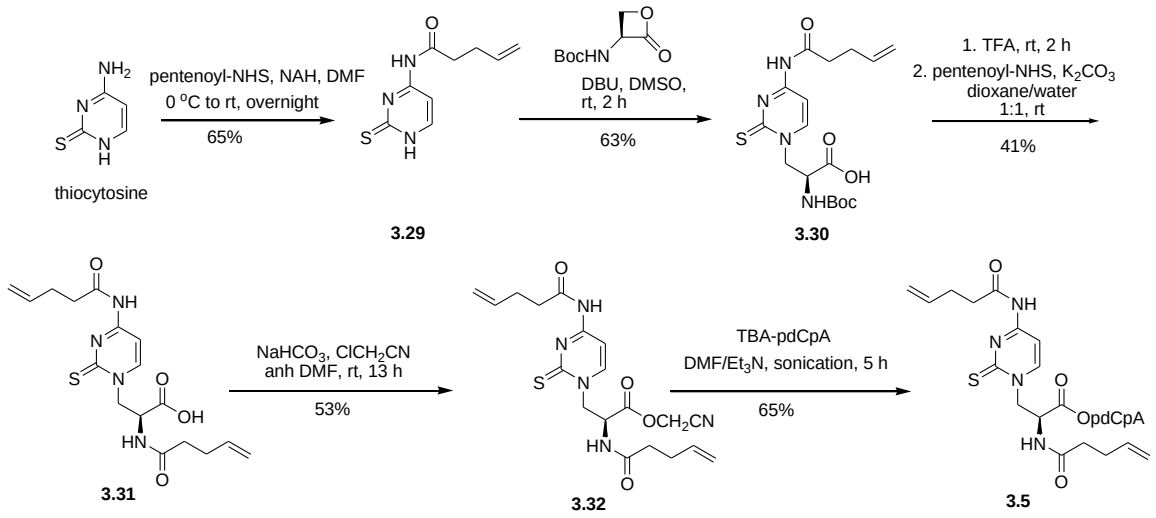
which was activated as cyanomethyl ester **3.28** in 31% overall yield. Treatment of cyanomethyl ester **3.28** with the tris(tetrabutylammonium) salt of pdCpA in anhydrous DMF afforded pdCpA ester **3.4** in 46% yield.



Scheme 3.4. Synthesis of **ANA-4** and its Aminoacyl-pdCpA.

The synthesis of the aminoacylated pdCpA derivative of **ANA-5** was accomplished starting from commercially available thiocytosine (Scheme 3.5). Pentenoyl protection of thiocytosine afforded compound **3.29**, the latter of which underwent coupling with *N*-Boc-L-serine β-lactone to afford compound **3.30** in 63% yield. Boc deprotection and subsequent protection using 4-pentenoic acid succinimide ester in the

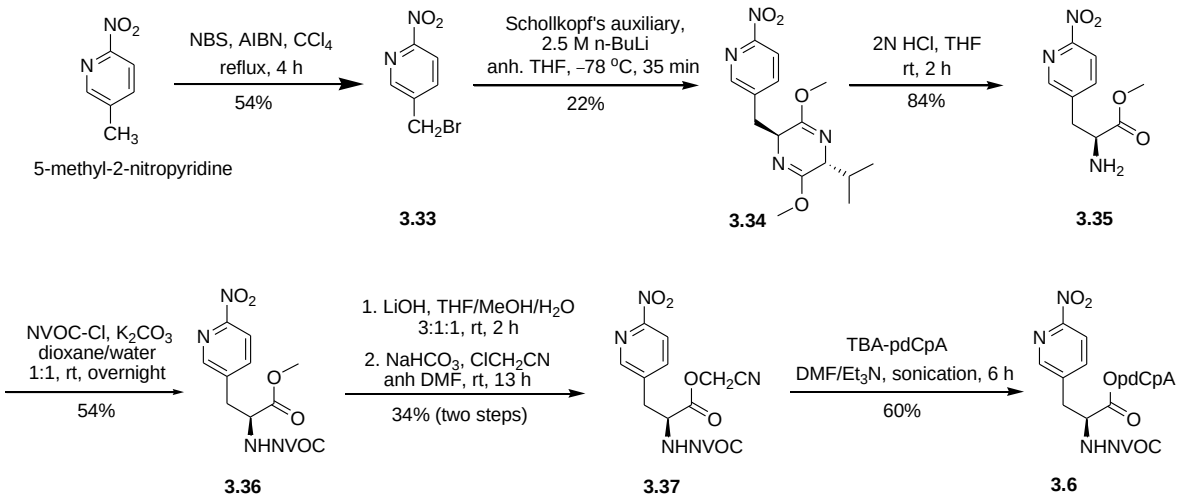
presence of K_2CO_3 afforded dipentenoyl amide **3.31** in 41% yield over two steps. Free acid **3.31** was activated as the cyanomethyl ester **3.32** in 53% yield. The pdCpA derivative of **ANA-5** was prepared from cyanomethyl ester **3.32** in 65% yield.



Scheme 3.5. Synthesis of **ANA-5** and its Aminoacyl-pdCpA.

The synthesis of the aminoacylated pdCpA derivative of amino acid **ANA-6** (Scheme 3.6) was accomplished starting from commercially available 5-methyl-2-nitropyridine which underwent bromination with NBS to afford **3.33**. Regioselective lithiation of the Schöllkopf chiral auxiliary with *n*-BuLi, followed by treatment with bromide **3.33** at ⁻78 °C afforded adduct **3.34** as a single diastereomer in 22% yield. Mild hydrolysis with 2 N HCl afforded the α-substituted amino acid methyl ester **3.35** in 84% yield. NVOC protection of free amine **3.35** afforded NVOC-carbamate **3.36** in 54% yield. Methyl ester **3.36** was hydrolyzed to afford the free acid, the latter of which was treated with chloroacetonitrile to afford the desired cyanomethyl ester **3.37** in 34% overall yield.

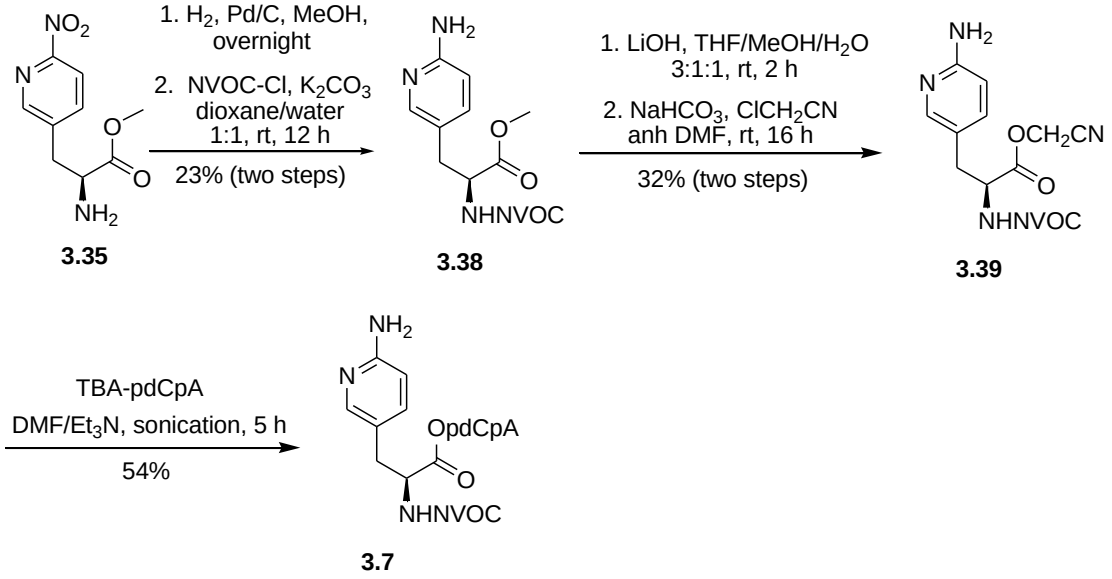
Treatment of the cyanomethyl ester **3.37** with a solution of the tris(tetrabutylammonium) salt of pdCpA in anhydrous DMF gave the aminoacylated pdCpA **3.6** in 60% yield.



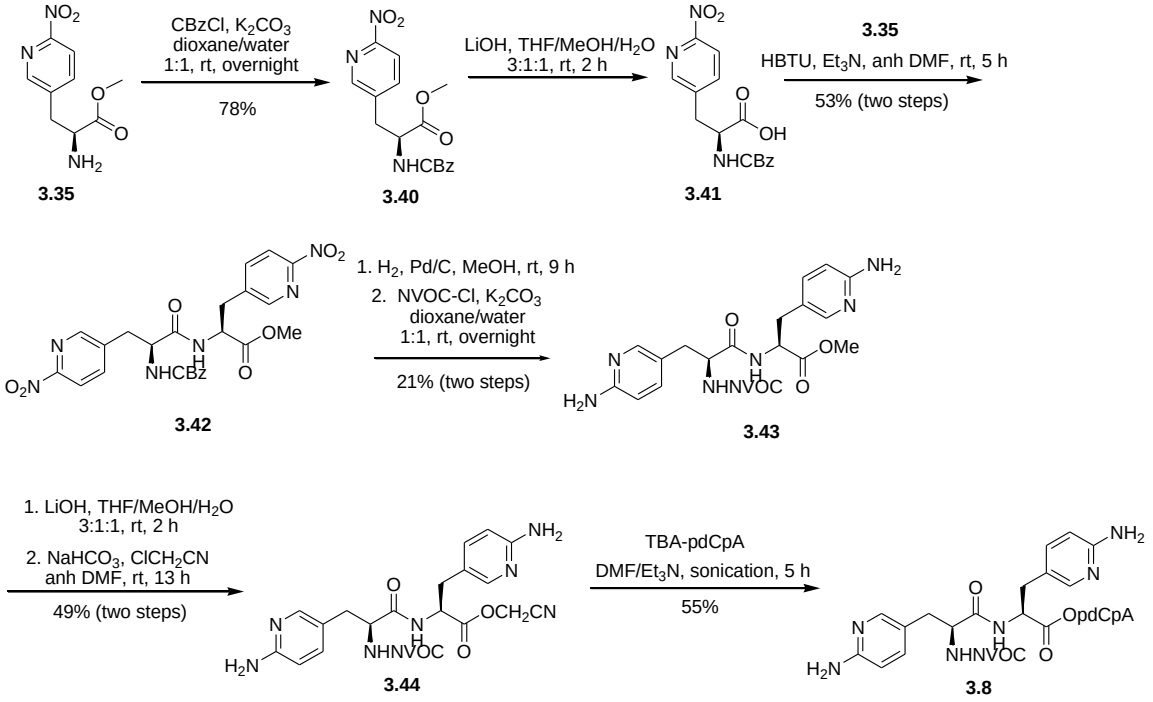
Scheme 3.6. Synthesis of **ANA-6** and its Aminoacyl-pdCpA.

The synthesis of the aminoacylated pdCpA derivative of amino acid **ANA-7** was accomplished starting from the intermediate **3.35** (Scheme 3.7). Nitro group reduction and subsequent NVOC protection afforded NVOC-carbamate **3.38** in 23% yield over two steps. NVOC methyl ester **3.38** was hydrolyzed to afford the free acid which was treated with chloroacetonitrile to afford the cyanomethyl ester **3.39** in 32% overall yield. Treatment of cyanomethyl ester **3.39** with the tris(tetrabutylammonium) salt of pdCpA in anhydrous DMF afforded pdCpA ester **3.7** in 54% yield.

The synthesis of the pdCpA derivative of amino acid **ANA-8** commenced with CBz protection of the intermediate **3.35** (Scheme 3.8). Methyl ester **3.40** was subjected to saponification in the presence of aqueous LiOH to produce **3.41**. This CBz protected acid was subsequently condensed with **3.35** in the presence of HBTU and Et_3N to afford the



Scheme 3.7. Synthesis of **ANA-7** and its Aminoacyl-pdCpA.

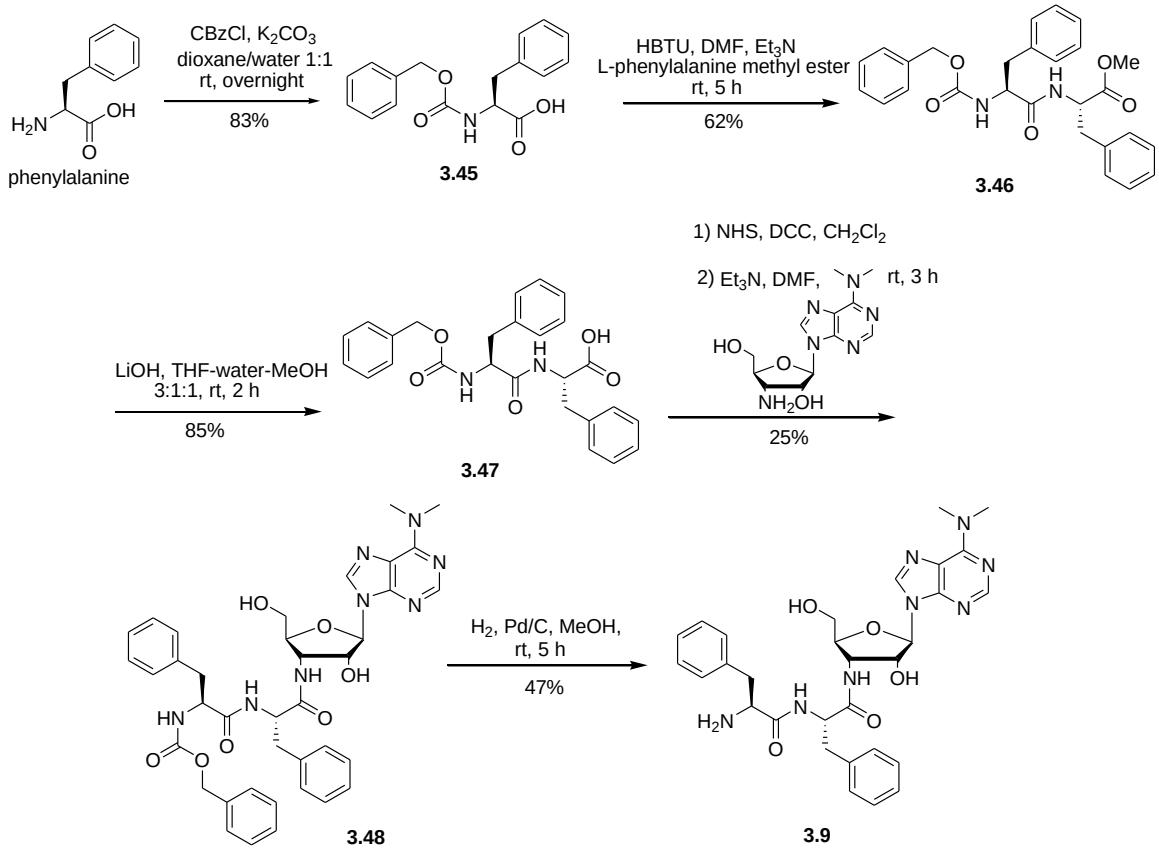


Scheme 3.8. Synthesis of **ANA-8** and its Aminoacyl-pdCpA.

N-protected dipeptide methyl ester **3.42** in 53% overall yield.[168] CBz deprotection and subsequent NVOC protection afforded NVOC-carbamate **3.43** in 21% yield over two steps. *N*-NVOC dipeptide methyl ester **3.43** was hydrolyzed to afford the free acid which was converted to the corresponding cyanomethyl ester **3.44** in 49% overall yield. Treatment of cyanomethyl ester **3.44** with the tris(tetrabutylammonium) salt of pdCpA in anhydrous DMF afforded dipeptidyl-pdCpA ester **3.8** in 55% yield.

Synthesis of Dipeptidylpuromycin 3.9

The synthesis of the dipeptidylpuromycin **3.9** commenced with the CBz protection of phenylalanine (Scheme 3.9). CBz-protected phenylalanine (**3.45**) was subsequently condensed with phenylalanine methyl ester in the presence of HBTU and DIPEA to afford the *N*-protected dipeptide methyl ester **3.46** in 62% yield (Scheme 3.9). The methyl ester was saponified in the presence of aqueous LiOH to produce **3.47** in 85% yield. Dipeptide acid **3.47** was treated with NHS in the presence of DCC to form *N*-hydroxysuccinimide ester, which was condensed with puromycin aminonucleoside to afford CBz-protected dipeptidylpuromycin **3.48** (Scheme 3.9).[168] Dipeptidylpuromycin **3.9** was then prepared in 47% yield from **3.48** by hydrogenolysis over Pd/C.



Scheme 3.9. Synthesis of Dipeptidylpuromycin **3.9**.

Activation of Suppressor $tRNA_{CUA}$ and Synthesis of Modified RRM1s

The individual *N*-protected aminoacylated pdCpA derivatives were ligated to a suppressor $tRNA_{CUA}$ lacking its 3′-terminal cytidine and adenosine residues ($tRNA_{CUA}$-C_{OH}) via the agency of T4 RNA ligase. The *N*-NVOC and *N*-pentenoyl protected misacylated tRNAs were deprotected by exposure to high intensity UV light at 4 ^{o}C and by treatment with aqueous iodine, respectively, to afford the activated tRNAs having **ANA**s with free α-amines. The aminoacyl-tRNAs so obtained were employed in an *in vitro* cell free transcription-translation system, which was programmed with pETRRM1-24 plasmid containing a TAG codon at the position corresponding to residue His24 of

RRM1. The incorporation of all the alanyl nucleobase amino acids into position 24 was evaluated via denaturing PAGE analysis and the incorporation yields were 5% to 71% as compared to the wild-type RRM1 (Table 3.1). It was found that four of the alanyl nucleobase amino acids (**ANA-1**, **ANA-2**, **ANA-6** and **ANA-7**) can suppress TAG codon for full size RRM1 synthesis with good suppression yields ranging from 23% to 71% (Table 3.1 and Figure 3.8) compared to wild-type RRM1. To incorporate dipeptide **ANA-8**, S30 010328R4, having modified ribosomes, selected against Phe-Gly-puromycin[168] was used and the incorporation yield was 5% to as compared to wild type.

Table 3.1. Expression Yields of RRM1s Modified at Position 24. The experiment was performed by Dr. Larisa Dedkova.

RRM1	position 24 (His) yield (%)
wild-type	100
ANA-1	23
ANA-2	24
ANA-3	6
ANA-4	6
ANA-5	6
ANA-6	35
ANA-7	71
ANA-8[a]	5

[a] S30 010328R4, having modified ribosome was used

Since, **ANA-7** was best among the series of alanyl nucleobase amino acids for suppressing a TAG codon for full size RRM1 synthesis, its aminoacyl-tRNA (**ANA7**-$tRNA_{CUA}$) was employed in an *in vitro* cell free transcription-translation system, which was programmed with pETRRM1-26 plasmid (containing TAG codon at the position corresponding to residue Arg26 of RRM1) or pETRRM1-2426 plasmid in which CAC (His24) and CGT (Arg26) both these codons simultaneously were substituted by TAG codon during PCR mutagenesis. The suppression efficiencies, relative to RRM1 synthesized from the wild-type gene, were 54% and 6%, respectively (Figure 3.9).

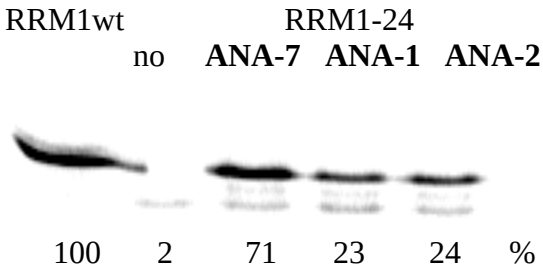


Figure 3.8. SDS-polyacrylamide gel analysis of translation of RRM1 from wild type (RRM1wt) and modified RRM1 mRNAs in the absence (no) and in the presence of tRNAs with **ANA7**, **ANA1** and **ANA2** respectively.

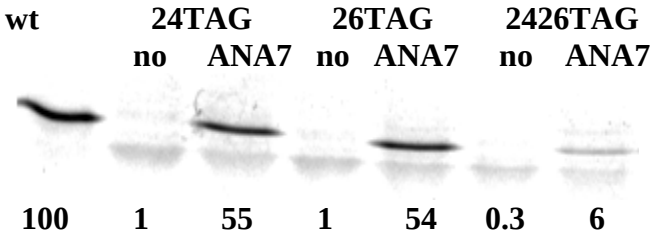


Figure 3.9. SDS-polyacrylamide gel electrophoresis of samples, obtained during *in vitro* translation of RRM1 from wild type (wt) and mutant (TAG codon in positions corresponding to His24 (24TAG); Arg26 (26TAG) and both His24 and Arg26 (2426TAG)) genes in the absence (no) and in the presence of **ANA7**-$tRNA_{CUA}$. The experiment was performed by Dr. Larisa Dedkova.

Electrophoretic mobility shift assay (EMSA)

The binding interaction between modified RRM1 and an i-motif DNA was studied and compared to wild-type RRM1 by employing a gel shift assay, EMSA.[169] RRM1 protein samples containing **ANA-7** at position 24 or 26, or at both positions, were tested in this assay and their binding ability was compared (Figure 3.10). It was shown that substitution of His24 or Arg26 by **ANA-7** has resulted in a 1.5-2 fold increased ability to form a complex with the 39 nt oligonucleotide 5' CAGCCCCGCTCCCGCCCCCTTCCTCCCGCGCCCGCCCCT 3', corresponding to

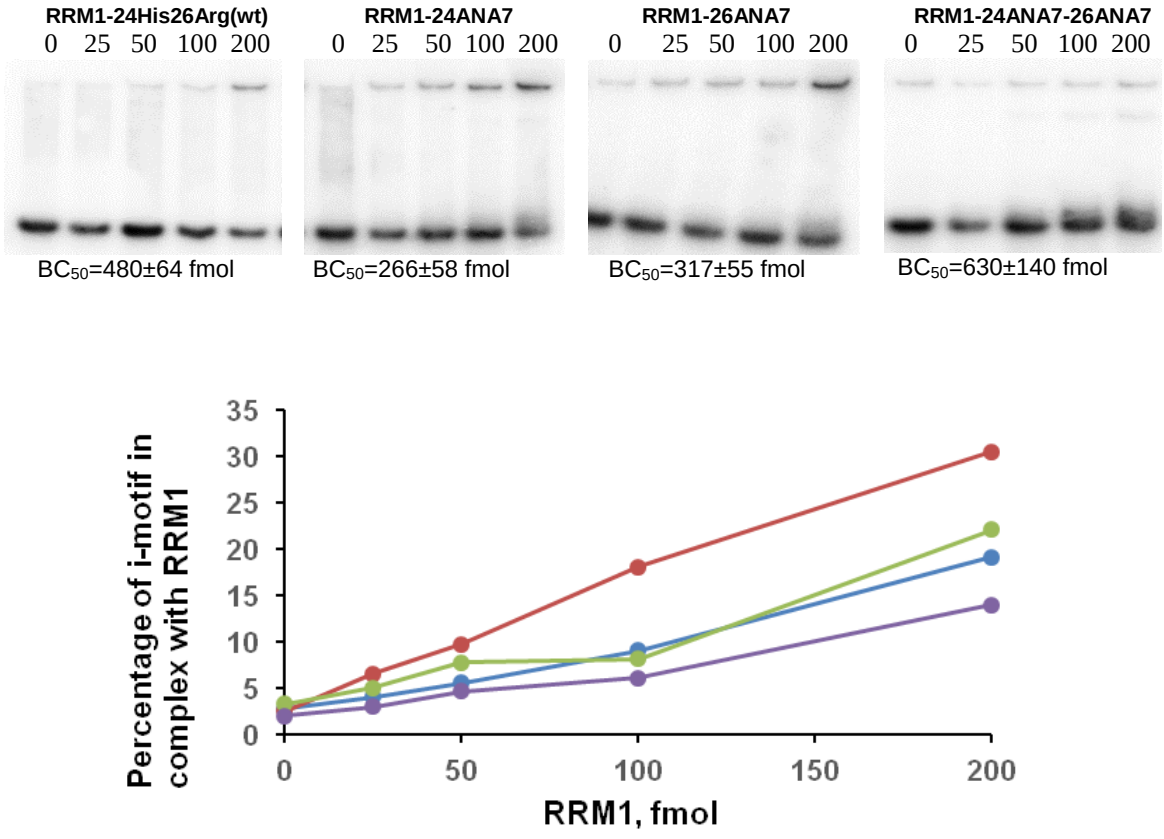


Figure 3.10. EMSA comparison of *BCL2* i-motif DNA binding ability of different samples of RRM1 at varying concentration (wild-type RRM1: blue, RRM1-24-**ANA-7**: red, RRM1-26-**ANA-7**: green, RRM1-2426-**ANA-7**: purple). The experiment was performed by Dr. Larisa Dedkova.

BCL2 i-motif, whereas substitution of both the positions simultaneously has decreased the ability of RRM1 to bind to the i-motif DNA (Figure 3.10). Comparison of binding activity of control and analyzed samples was expressed in BC_{50} (concentration at which 50% of the protein was complexed with the i-motif DNA).

4.3. Discussion

As part of our ongoing efforts to develop molecules which can bind with high sequence specificity to a chosen target in a gene sequence, a series of alanyl nucleobase amino acids was synthesized. Their aminoacyl-tRNA derivatives were used for their incorporation into predetermined positions of RRM1 by suppression of nonsense codons. Boc-protected alanyl nucleobase amino acids were obtained by ring opening of *N*-Boc-L-serine β-lactone with the DNA bases (Scheme 3.1 – Scheme 3.5). The yields are quite low due to their low solubility in DMSO, regioselectivity problems (S_N2 vs. carbonyl attack on the serine lactone and N^7 vs. N^9 alkylation) and racemization.[162] Uracil and thymine are the only nucleobases that did not require protection. The synthesis of the aminoacylated pdCpA derivative of cytosinylalanine (**ANA-1**) commenced with CBz protection of cytosine (Scheme 3.1). Similarly, for the synthesis of the aminoacylated pdCpA derivative of thiocytosinylalanine (**ANA-5**) the starting material, thiocytosine, was protected as its *N*-pentenoylamide (Scheme 3.5). The asymmetric syntheses of the **ANA-6** – **ANA-8** were challenging and started with the asymmetric synthesis using Ni chiral auxiliary.[47] Several attempts were made to alkylate the Ni chiral auxiliary with bromide **3.33** but none of them resulted in successful asymmetric synthesis in reasonable yield. Finally, lithiation of the Schöllkopf chiral auxiliary with *n*-BuLi, followed by

treatment with bromide **3.33** at ⁻78 °C, afforded the adduct **3.34** as a single diastereomer in 22% yield (Scheme 3.6). Despite extensive efforts to improve the yield of the asymmetric synthesis, so far the route involving the Schöllkopf reagent has turned out to be the most efficient. Cyanomethyl esters of the *N*-protected amino acids were coupled with pdCpA TBA salt to give dinucleotide esters (**3.1**–**3.8**) which was obtained as colorless solids after reversed-phase HPLC purification. Misacylated tRNAs were produced by ligating abbreviated tRNA-C_{OH}, with pdCpA derivatives **3.1**–**3.8** using T4 tRNA ligase. The activated tRNAs were deprotected by UV irradiation or iodine treatment to afford free aminoacyl-tRNAs.

Based on calculations (done by Dr. Sashka Daskalova) using different computer programs, positions His24 and Arg26 of RRM1 which participate in binding of the protein RRM1 with the *BCL2* i-motif were chosen for mutagenesis. Alanyl nucleobase amino acids (**ANA**s) were incorporated into position 24 of RRM1 and **ANA-7**, exhibiting the best suppression yield, was further used for incorporation into 26 position of RRM1. As shown in Figure 3.10, RRM1-24-**ANA7** and RRM1-26-**ANA7**, both had stronger affinity (BC_{50}= 266 ± 58 fmol and 317 ± 55 fmol, respectively) for the i-motif DNA compared to wild-type RRM1 (BC_{50}= 480 ± 64 fmol). Arg26 probably plays a more important role (possibly through H-bonding and electrostatic interaction) in binding than His24. Therefore, RRM1-26-**ANA7** has decreased binding ability compared to RRM1-24-**ANA7**. Substitution of the two amino acids by **ANA-7** decreased the binding affinity significantly (BC_{50}= 630 ± 140 fmol), probably due to perturbation of the secondary structure used for DNA recognition.

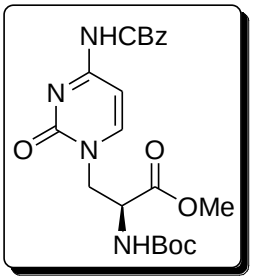
To incorporate **ANA-8**, S30 010328R4, having modified ribosomes, selected against Phe-Gly-puromycin[168] was used for incorporation, but yield was only 5% as compared to the wild type. Therefore, to improve the incorporation yield, puromycin **3.9** has been synthesized anticipating that it will enable the selection of ribosomes that will accept dipeptide **ANA-8** more efficiently during protein synthesis.

3.4. Experimental Procedures

The chemicals used were purchased from Aldrich Chemical Co., Sigma Chemical Co. or Combi-Blocks and were used without further purification. Anhydrous DMSO and DMF were used as purchased. Tetrahydrofuran and dichloromethane were distilled from sodium/benzophenone and calcium hydride, respectively. The tris-(tetrabutylammonium) salt of pdCpA was prepared by passing pdCpA through the activated TBA form of Dowex 50W×8 (200-400 mesh). All experiments requiring anhydrous conditions were conducted in flame-dried glassware fitted with rubber septa under a positive pressure of dry nitrogen, unless otherwise noted. Reactions were performed at room temperature unless indicated otherwise. Analytical thin-layer chromatography was performed using glass plates pre-coated with silica gel (0.25 mm, 60 Å pore size, 230-400 mesh, Silicycle) impregnated with a fluorescent indicator (254 nm). TLC plates were visualized by exposure to ultraviolet light (UV). Flash column chromatography was performed employing silica gel (60 Å pore size, 40-63 μm, standard grade, Silicycle). An acetone bath was cooled to the appropriate temperature by addition of small portions of dry ice.

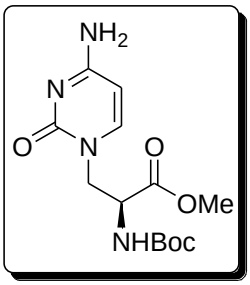
^{1}H NMR and ^{13}C NMR spectra were recorded on Varian INOVA 400 (400 MHz) and Varian INOVA 500 (500 MHz) spectrometers at 25 °C. Proton chemical shifts are

expressed in parts per million (ppm, δ scale) and are referenced to residual protium in the NMR solvent ($CDCl_3$ or DMSO-d_6 or CD_3OD). Splitting patterns are designated as follows: s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or the Michigan State University Mass Spectrometry Facility. HPLC purification was performed with a Waters 600 pump coupled with a Varian ProStar 340 detector and a Grace Econosil C_{18} column (250 x 10 mm, 5 μm). The tetra-*n*-butylammonium (TBA) salt of pdCpA was prepared using Dowex 50WX8, 200–400 mesh activated in its TBA form.

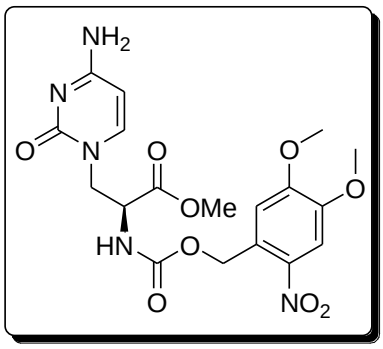


Methyl (*S*)-3-(4-(Benzyloxycarbonylamino)-2-oxopyrimidin-1(2*H*)-yl)-2-(*tert* butoxycarbonylamino)propionate (3.12). To a stirred suspension of 1.44 g (36.0 mmol) of NaH (60% in mineral oil) in 50 ml of DMF at 0 °C was added 1.00 g (9.01 mmol) of cytosine. The reaction mixture was stirred at 0 °C for 1 h, then 1.35 mL (9.50 mmol) of ClCOOBn was added. The reaction mixture was stirred at room temperature for 14 h, then diluted with 100 mL of H_2O and ice. After neutralization with 5 N HCl, a colorless precipitate formed, and was filtered, washed with five 50-mL portions of H_2O and dried to give **3.10** as a colorless powder: yield 1.71 g (74%); mass spectrum (APCI), *m*/*z* 246.0890 $(M+H)^+$ ($C_{12}H_{12}N_3O_3$ requires *m*/*z* 246.0879).

To a stirred suspension containing 250 mg (1.02 mmol) of compound **3.10** in 5 mL of DMSO was added 0.18 mL (183 mg, 1.20 mmol) of DBU. Within 15 min, a solution containing 220 mg (1.20 mmol) of *N*-(*tert*-butoxycarbonyl)-L-serine β-lactone in 5 mL of DMSO was added. The reaction mixture was stirred at room temperature for 2 h under argon atmosphere, then diluted with 10 mL of 0.5 N HCl and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave **3.11** as a colorless solid: yield 246 mg (56%); silica gel TLC R_f 0.31 (5:1 ethyl acetate–methanol). To a cooled (0 °C) solution containing 246 mg (0.60 mmol) of **3.11** in 5 mL of anhydrous MeOH was added dropwise 0.04 mL (67.7 mg, 0.60 mmol) of $SOCl_2$. The reaction mixture was allowed to warm slowly to room temperature with stirring for 2 h, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave **3.12** as a colorless solid: yield 165 mg (65%); silica gel TLC R_f 0.72 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.35 (s, 9H), 3.64 (s, 3H), 4.01-4.60 (m, 3H), 5.19 (s, 2H), 5.86-5.96 (m, 4H), 6.29 (br s, 1H), 7.17 (s, 1H), 7.55 (s, 1H), 7.75 (s, 1H) and 8.70 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 28.2, 50.8, 52.1, 52.8, 67.9, 80.5, 94.8, 128.3, 128.58, 128.63, 135.09, 149.7, 150.5, 152.5, 155.5, 162.9 and 170.4; mass spectrum (APCI), *m*/*z* 447.1890 $(M+H)^+$ ($C_{21}H_{27}N_4O_7$ requires *m*/*z* 447.1880).

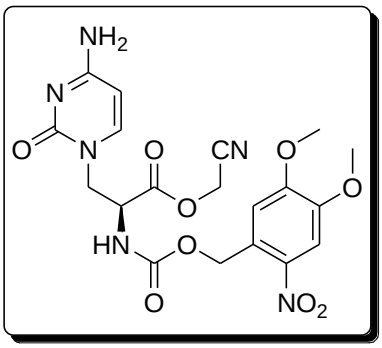


Methyl (*S*)-3-(4-Amino-2-oxopyrimidin-1(2*H*)-yl)-2-(*tert*-butoxycarbonylamino)propionate (3.13). To a solution containing 164 mg (0.36 mmol) of **3.12** in 5 mL of MeOH was added catalytic amount of 10% Pd/C and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and the filtrate was concentrated under diminished pressure and purified by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 ethyl acetate–methanol gave **3.13** as a colorless solid: yield 98.0 mg (85%); silica gel TLC R_f 0.11 (5:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 1.37 (s, 9H), 3.71-3.73 (m, 4H), 4.34-4.59 (m, 2H), 5.83 (d, 1H, J = 7.2 Hz) and 7.44 (d, 1H, J = 7.2 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 28.6, 53.0, 53.2, 53.5, 53.6, 80.9, 95.8, 147.9, 148.2, 157.6, 158.7, 167.9 and 172.0; mass spectrum (APCI), *m*/*z* 313.1506 $(M+H)^+$ ($C_{13}H_{21}N_4O_5$ requires *m*/*z* 313.1512).



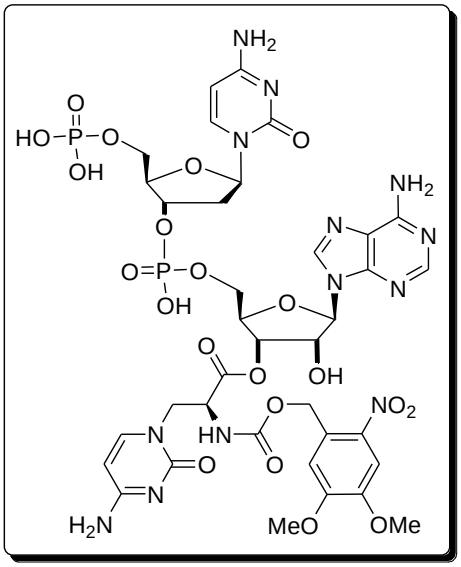
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(4-amino-2-oxopyrimidin-1(2*H*)-yl)propionate (3.14). To a stirred solution containing 97.0 mg

(0.31 mmol) of **3.13** in 2 mL of anhydrous CH_2Cl_2 was added 0.24 mL (353 mg, 3.10 mmol) of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 2 h and then concentrated under diminished pressure. To a stirred solution containing Boc-deprotection product in 2 mL of 1:1 dioxane–water was added 129 mg (0.93 mmol) of K_2CO_3 followed by 85.5 mg (0.31 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 14 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave **3.14** as a light yellow solid: yield 49.2 mg (35% for two steps); silica gel TLC R_f 0.25 (5:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 3.71 (s, 3H), 3.84 (s, 3H), 3.90 (s, 3H), 4.35-4.39 (m, 1H), 4.77-4.81 (m, 2H), 5.31 (ABq, 2H, J = 10.8 Hz), 5.83 (d, 1H, J = 7.2 Hz), 7.04 (s, 1H), 7.48 (d, 2H, J = 7.2 Hz) and 7.58 (s, 1H); mass spectrum (APCI), m/z 452.1413 $(M+H)^+$ ($C_{18}H_{22}N_5O_9$ requires m/z 452.1417).



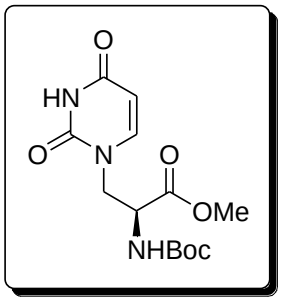
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(4-amino-2-oxopyrimidin-1(2*H*)-yl)propionate (3.15). To a stirred solution containing 14.0 mg (0.03 mmol) of **3.14** in 1 mL of 1:3:1 water–THF–methanol was added 90.0 µL (0.09 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and

then concentrated under diminished pressure. The residue was dissolved in 1 mL of anhydrous DMF under argon and 25.0 mg (0.30 mmol) of $NaHCO_3$ was added followed by 4.0 µL (5.0 mg, 0.06 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for overnight, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave **3.15** as a light yellow solid: yield 5.01 mg (34% for two steps); silica gel TLC R_f 0.26 (5:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 3.89 (s, 3H), 3.94-3.96 (m, 4H), 4.36-4.41 (m, 1H), 4.71-4.75 (m, 1H), 4.92 (s, 2H), 5.38-5.48 (m, 2H), 5.84 (br s, 1H), 7.12 (s, 1H), 7.48 (d, 2H, J = 7.6 Hz) and 7.71 (s, 1H); mass spectrum (APCI), m/z 477.1377 $(M+H)^+$ ($C_{19}H_{21}N_6O_9$ requires m/z 477.1370).



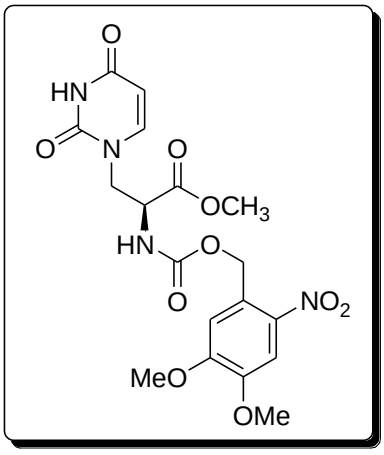
((*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(4-amino-2-oxopyrimidin-1(2*H*)-yl)propionic Acid pdCpA Ester (3.1). To a solution containing

5.20 mg (4.00 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 10.0 mg (21.0 μmol) of **3.15**. The reaction mixture was sonicated for 5 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 20.1 min. The fractions containing the product were lyophilized to afford **3.1** as a colorless solid: yield 1.01 mg (25%); mass spectrum (ESI), *m/z* 1054.2106 $(M-H)^-$ ($C_{36}H_{42}N_{13}O_{21}P_2$ requires *m/z* 1054.2093).



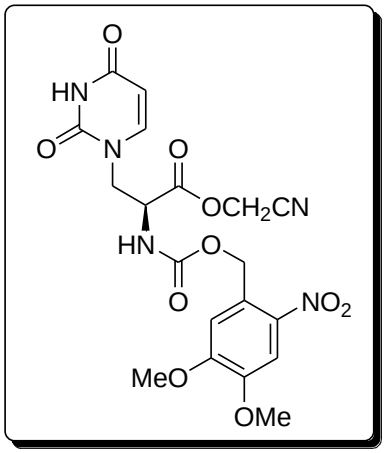
Methyl (*S*)-2-(*tert*-Butoxycarbonyl)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propionate (3.17). To a stirred suspension containing 250 mg (2.23 mmol) of nucleobase uracil in 5 mL of DMSO was added 0.40 mL (407 mg, 2.68 mmol) of DBU. Within 15 min, a solution containing 491 mg (2.68 mmol) of *N*-(*tert*-butoxycarbonyl)-L-serine β-lactone in 5 mL of DMSO was added. The reaction mixture was stirred at room temperature for 2 h under argon, then diluted with 10 mL of 0.5 N HCl and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave **3.16** as a colorless solid: yield 273 mg (41%); silica gel TLC R_f 0.25 (5:1 ethyl acetate–methanol). To a cooled (0 °C) solution containing 246 mg (0.91 mmol) of **3.16** in 5 mL of anhydrous

MeOH was added dropwise 0.07 mL (71.2 mg, 0.60 mmol) of $SOCl_2$. The reaction mixture was allowed to warm slowly to room temperature with stirring for 2 h, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave **3.17** as a colorless solid: yield 214 mg (75%); silica gel TLC R_f 0.81 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.36 (s, 9H), 3.74 (s, 3H), 4.20-4.49 (m, 3H), 5.64 (s, 1H), 5.73 (s, 1H), 7.17 (s, 1H) and 10.10 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.3, 49.7, 52.4, 53.1, 80.8, 102.2, 145.2, 151.4, 155.4, 164.2 and 170.6; mass spectrum (APCI), *m*/*z* 314.1345 $(M+H)^+$ ($C_{13}H_{20}N_3O_6$ requires *m*/*z* 314.1352).



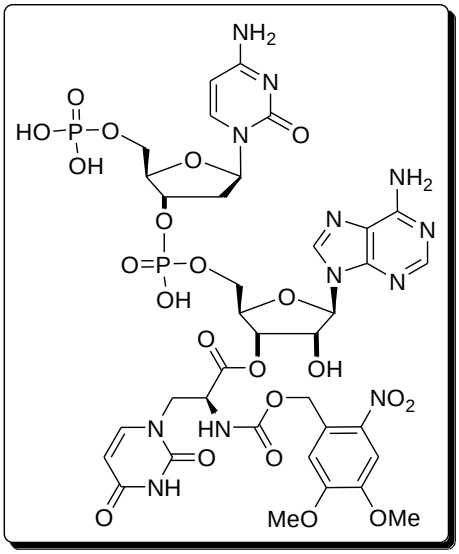
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propionate (3.18). To a stirred solution containing 94.0 mg (0.30 mmol) of **3.17** in 2 mL of anhydrous CH_2Cl_2 was added 0.23 mL (342 mg, 3.00 mmol) of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 2 h and then concentrated under diminished pressure. To a stirred solution containing Boc-deprotection product in 2 mL of 1:1 dioxane–water was added 124 mg (0.90 mmol) of

K_2CO_3 followed by 82.7 mg (0.30 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 14 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave **3.18** as a light yellow solid: yield 73.3 mg (54% for two steps); silica gel TLC R_f 0.60 (10:1 ethyl acetate–methanol); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.66 (s, 3H), 3.73-3.78 (m, 1H), 3.87 (s, 3H), 3.91 (s, 3H), 4.19-4.23 (m, 1H), 4.43-4.45 (m, 1H), 5.34 (ABq, 2H, J = 14.5 Hz), 5.46 (d, 1H, J = 7.5 Hz), 7.15 (s, 1H), 7.48 (d, 1H, J = 8.0 Hz), 7.70 (s, 1H) and 8.10 (d, 1H, J = 8.5 Hz); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 48.0, 52.1, 52.3, 56.0, 56.1, 62.8, 100.7, 108.1, 110.5, 127.3, 139.2, 145.8, 147.7, 150.9, 153.3, 155.5, 163.5 and 170.0; mass spectrum (APCI), m/z 453.1259 $(M+H)^+$ ($C_{18}H_{21}N_4O_{10}$ requires m/z 453.1257).



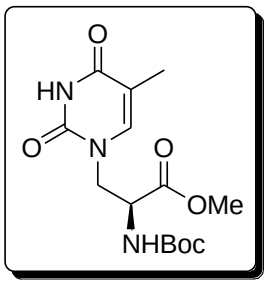
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propionate (3.19). To a stirred solution containing 22.7 mg (0.05 mmol) of **3.18** in 1 mL of 1:3:1 water–THF–methanol was added 150 µL (0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was redissolved into 1 mL of

anhydrous DMF and 25.0 mg (0.30 mmol) of $NaHCO_3$ was added followed by 10.0 μL (11.3 mg, 0.15 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for overnight, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave the desired product **3.19** as a yellow solid: yield 7.17 mg (30% for two steps); silica gel TLC R_f 0.75 (5:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 3.91 (s, 3H), 3.99 (s, 3H), 4.33-4.36 (m, 2H), 4.67-4.71 (m, 2H), 4.87-4.93 (m, 2H), 5.41-5.51 (m, 2H), 5.67 (d, 1H, J = 7.6 Hz), 7.13-7.15 (m, 1H), 7.33-7.43 (m, 1H), 7.73 (s, 1H) and 7.97 (s, 1H); mass spectrum (APCI), m/z 478.1213 $(M+H)^+$ ($C_{19}H_{20}N_5O_{10}$ requires m/z 478.1210).



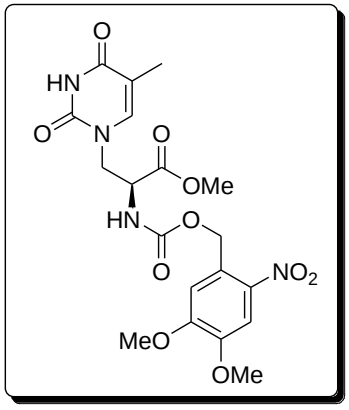
((*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propionic Acid pdCpA Ester (3.2). To a solution containing 5.20 mg (4.00 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 10.0 mg (21.0 μmol) of **3.19**. The reaction

mixture was sonicated for 5 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 18.6 min. The fractions containing the product were lyophilized to afford **3.2** as a colorless solid: yield 2.02 mg (46%); mass spectrum (ESI), *m/z* 1055.1943 $(M-H)^-$ ($C_{36}H_{41}N_{12}O_{22}P_2$ requires *m/z* 1055.1934).



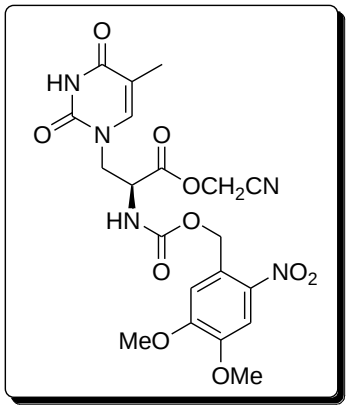
Methyl (*S*)-2-(*tert*-Butoxycarbonylamino)-3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propionate (3.21). To a stirred suspension containing 280 mg (2.23 mmol) of thymine in 5 mL of DMSO was added 0.40 mL (407 mg, 2.68 mmol) of DBU. Within 15 min, a solution containing 491 mg (2.68 mmol) of *N*-(*tert*-butoxycarbonyl)-L-serine β-lactone in 5 mL of DMSO was added. The reaction mixture was stirred at room temperature for 2 h under argon, then diluted with 10 mL of 0.5 N HCl and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave **3.20** as a colorless solid: yield 230 mg (33%); silica gel TLC R_f 0.30 (5:1 ethyl acetate–methanol). To a cooled (0 °C) solution containing 230 mg (0.73 mmol) of **3.20** in 5 mL of anhydrous MeOH was added dropwise 0.05 mL (87.4 mg, 0.73 mmol) of $SOCl_2$. The reaction mixture was allowed to warm slowly to room temperature with

stirring for 2 h, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave **3.21** as a colorless solid: yield 156 mg (65%); silica gel TLC R_f 0.81 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.41 (s, 9H), 1.88 (s, 3H), 3.78 (s, 3H), 4.03-4.20 (m, 2H), 4.47-4.50 (m, 1H), 5.55 (s, 1H), 6.98 (s, 1H) and 9.34 (br s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 12.4, 28.6, 50.0, 50.3, 53.1, 80.9, 110.7, 143.0, 152.6, 157.2, 166.5 and 171.4; mass spectrum (APCI), *m*/*z* 328.1500 $(M+H)^+$ ($C_{14}H_{22}N_3O_6$ requires *m*/*z* 328.1509).



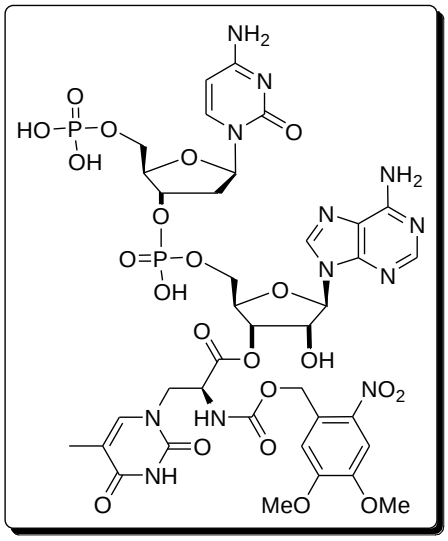
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propionate (3.22). To a stirred solution containing 49.2 mg (0.15 mmol) of **3.21** in 1.5 mL of anhydrous CH_2Cl_2 was added 0.12 mL (171 mg, 1.50 mmol) of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 2 h and then concentrated under diminished pressure. To a stirred solution containing Boc-deprotection product in 2 mL of 1:1 dioxane–water was added 124 mg (0.90 mmol) of K_2CO_3 followed by 41.4 mg (0.15 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 14 h under argon, then diluted with

50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave **3.22** as a light yellow solid: yield 32.7 mg (47% for two steps); silica gel TLC R_f 0.75 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.88 (s, 3H), 3.78 (s, 3H), 3.95 (s, 3H), 3.99 (s, 3H), 4.13-4.15 (m, 1H), 4.34-4.37 (m, 1H), 4.57-4.59 (m, 1H), 5.49 (s, 2H), 6.03 (br s, 1H), 6.99 (s, 2H), 7.69 (s, 1H) and 9.08 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 12.3, 49.2, 53.2, 53.4, 56.5, 56.6, 64.3, 108.3, 110.4, 111.2, 127.5, 139.8, 140.9, 148.3, 151.7, 153.7, 155.7, 164.4 and 170.0; mass spectrum (APCI), *m*/*z* 467.1411 $(M+H)^+$ ($C_{19}H_{23}N_4O_{10}$ requires *m*/*z* 467.1414).



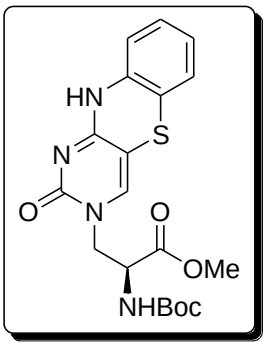
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propionate (3.23). To a stirred solution containing 23.3 mg (0.05 mmol) of **3.22** in 1 mL of 1:3:1 water–THF–methanol was added 150 μL (0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was redissolved into 1 mL of anhydrous DMF and 25.0 mg (0.30 mmol) of $NaHCO_3$ was added followed by 10.0 μL (11.3 mg, 0.15 mmol) of chloroacetonitrile. The reaction

mixture was stirred at 23 °C for overnight, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave the desired product **3.23** as a yellow solid: yield 8.59 mg (35% for two steps); silica gel TLC R_f 0.77 (5:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 1.83 (s, 3H), 3.91 (s, 3H), 3.96 (s, 3H), 4.28-4.34 (m, 2H), 4.70-4.73 (m, 1H), 4.92 (s, 3H), 5.44 (ABq, 2H, J = 14.8 Hz), 7.10 (s, 2H), 7.26 (s, 1H) and 7.70 (s, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 11.8, 47.7, 49.8, 51.9, 56.0, 56.2, 62.9, 108.1, 108.4, 110.6, 115.5, 127.1, 139.2, 141.5, 147.7, 150.9, 153.3, 155.5, 164.1 and 168.9; mass spectrum (APCI), *m*/*z* 492.1363 $(M+H)^+$ ($C_{20}H_{22}N_5O_{10}$ requires *m*/*z* 492.1366).



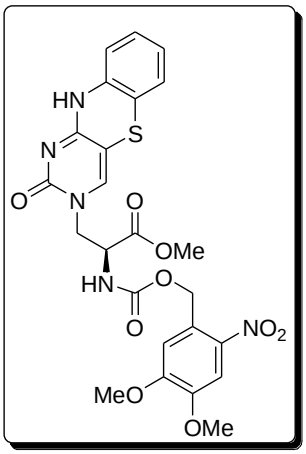
((*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propionic Acid pdCpA Ester (3.3). To a solution containing 5.20 mg (4.00 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 10.3 mg (21.0 μmol) of **3.23**. The reaction

mixture was sonicated for 5 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 20.3 min. The fractions containing the product were lyophilized to afford **3.3** as a colorless solid: yield 1.81 mg (45%); mass spectrum (ESI), *m/z* 1069.2089 $(M-H)^-$ ($C_{37}H_{43}N_{12}O_{22}P_2$ requires *m/z* 1069.2090).



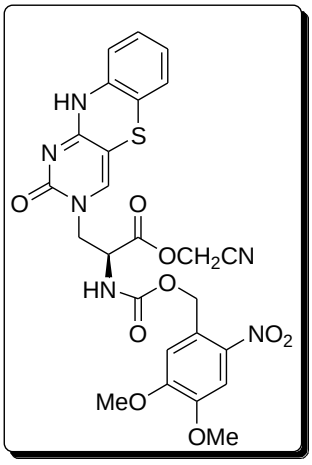
Methyl (*S*)-2-(*tert*-Butoxycarbonylamino)-3-(1,3-diaza-2-oxophenothiazinyl) propionate (3.26). To a stirred suspension containing 220 mg (1.01 mmol) of compound **3.24** in 5 mL of DMSO was added 0.18 mL (185 mg, 1.22 mmol) of DBU. Within 15 min, a solution containing 220 mg (1.20 mmol) of *N*-(*tert*-butoxycarbonyl)-L-serine β-lactone in 5 mL of DMSO was added. The reaction mixture was stirred at room temperature for 2 h under argon, then diluted with 10 mL of 0.5 N HCl and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave **3.25** as a yellow solid: yield 294 mg (59%); silica gel TLC R_f 0.34 (5:1 ethyl acetate–methanol). To a cooled (0 °C) solution containing 294 mg (0.73 mmol) of **3.25** in 5 mL of anhydrous MeOH was added dropwise 0.05 mL (86.6 mg, 0.73 mmol) of $SOCl_2$. The reaction

mixture was allowed to warm slowly to room temperature with stirring for 2 h, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave **3.26** as a colorless solid: yield 183 mg (70%); silica gel TLC R_f 0.75 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.42 (s, 9H), 3.75 (s, 3H), 4.14-4.18 (m, 2H), 4.50-4.51 (m, 1H), 5.92 (d, 1H, J = 6.4 Hz) and 6.87-7.13 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.4, 50.5, 52.9, 53.0, 80.4, 97.0, 116.1, 118.4, 124.5, 125.8, 127.6, 135.7, 138.9, 155.5, 155.8, 160.8 and 170.6; mass spectrum (APCI), *m*/*z* 419.1401 $(M+H)^+$ ($C_{19}H_{23}N_4O_5S$ requires *m*/*z* 419.1389).



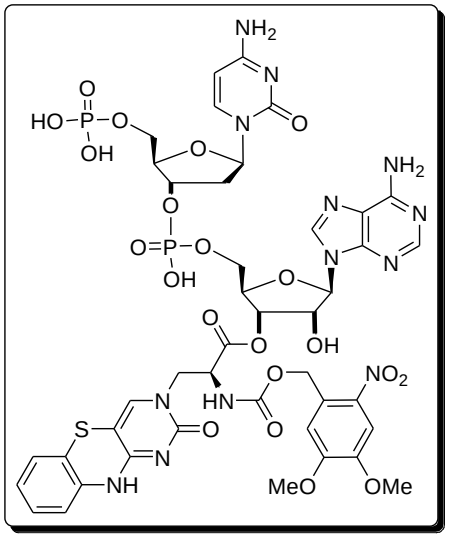
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1,3-diaza-2-oxophenothiazinyl)propionate (3.27). To a stirred solution containing 100 mg (0.24 mmol) of **3.26** in 2 mL of anhydrous CH_2Cl_2 was added 0.18 mL (272 mg, 2.40 mmol) of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 2 h and then concentrated under diminished pressure. To a stirred solution containing Boc-deprotection product in 2 mL of 1:1 dioxane–water was added 198 mg (1.44 mmol) of K_2CO_3 followed by 66.2 mg (0.24 mmol) of NVOC-Cl. The reaction mixture was stirred

at room temperature for 14 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave **3.27** as a light yellow solid: yield 53.3 mg (40% for two steps); silica gel TLC R_f 0.70 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.66 (s, 3H), 3.70-3.75 (m, 1H), 3.85 (s, 3H), 3.91 (s, 3H), 4.20-4.25 (m, 1H), 4.46-4.50 (m, 1H), 5.34 (ABq, 2H, J = 10.8 Hz), 6.90-6.92 (m, 2H), 6.98-7.08 (m, 2H), 7.16 (s, 1H), 7.52 (br s, 1H), 7.67 (s, 1H) and 8.09 (d, 1H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 49.4, 52.2, 52.3, 56.0, 56.1, 62.7, 93.4, 108.0, 110.2, 115.6, 123.8, 125.8, 127.3, 127.5, 136.17, 136.21, 139.0, 147.6, 153.3, 154.4, 155.5 and 170.2; mass spectrum (APCI), m/z 558.1303 $(M+H)^+$ ($C_{24}H_{24}N_5O_9S$ requires m/z 558.1294).



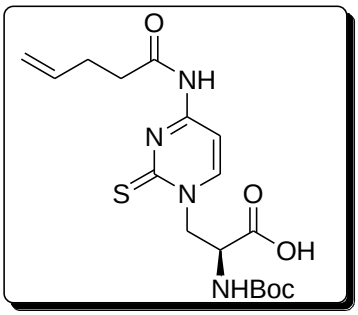
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1,3-diaza-2-oxophenothiazinyl)propionate (3.28). To a stirred solution containing 28.0 mg (0.05 mmol) of **3.27** in 1 mL of 1:3:1 water–THF–methanol was added 150 µL (0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then

concentrated under diminished pressure. The residue was redissolved into 1 mL of anhydrous DMF and 25.0 mg (0.30 mmol) of $NaHCO_3$ was added followed by 4.0 µL (5.0 mg, 0.06 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for overnight, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave **3.28** as a light yellow solid: yield 9.06 mg (31% for two steps); silica gel TLC R_f 0.71 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.59-3.61 (m, 2H), 3.87 (s, 3H), 3.96 (s, 3H), 4.32-4.36 (m, 1H), 4.92 (s, 2H), 5.43-5.49 (m, 2H), 6.81 (d, 2H, J = 6.4 Hz), 6.91 (br s, 1H), 7.02-7.05 (m, 1H), 7.13 (s, 1H), 7.22 (s, 1H), 7.69 (s, 1H) and 7.72 (s, 1H); mass spectrum (APCI), m/z 583.1232 $(M+H)^+$ ($C_{25}H_{23}N_6O_9S$ requires m/z 583.1247).



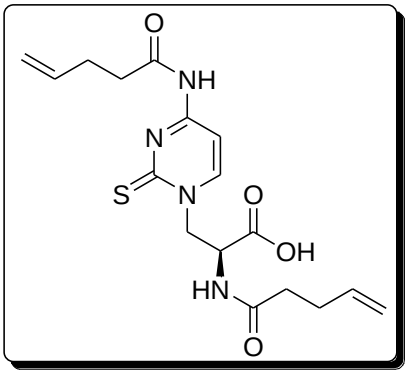
((*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(1,3-diaza-2-oxophenothiazinyl)propionic Acid pdCpA Ester (3.4). To a solution containing 5.20 mg (4.00 µmol) of pdCpA tetrabutylammonium salt in 100 µL of 9:1 anhydrous DMF–

triethylamine was added 12.2 mg (21.0 μmol) of **3.28**. The reaction mixture was sonicated for 5 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 24.9 min. The fractions containing the product were lyophilized to afford **3.4** as a colorless solid: yield 2.10 mg (46%); mass spectrum (ESI), *m/z* 1160.1956 $(M-H)^-$ ($C_{42}H_{44}N_{13}O_{21}SP_2$ requires *m/z* 1160.1971).

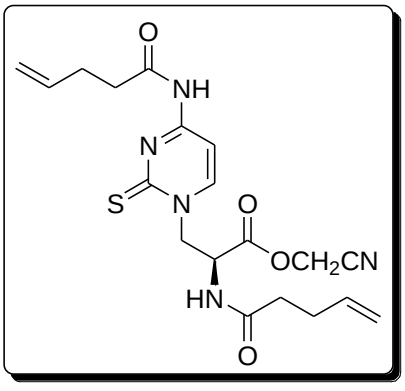


(*S*)-2-(*tert*-butoxycarbonylamino)-3-(4-pent-4-enamido-2-thioxopyrimidin-1(2*H*)-yl)propionic acid (3.30). To a stirred solution containing 1.44 g (36.0 mmol) of NaH (60% in mineral oil) in 30 mL of anhydrous DMF at 0 °C was added 1.14 g (9.00 mmol) of thiocytosine. The reaction mixture was stirred at 0 °C for 1 h under argon and then 1.87 g (9.50 mmol) of pentenoyl-NHS ester was added. The reaction mixture was stirred at 0 °C under argon for 3 h, diluted with 150 mL of brine and extracted with two 50-mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 10:1 ethyl acetate–methanol gave the desired product **3.29** as a yellow solid: yield 844 mg (65%); silica gel TLC R_f 0.42 (10:1 ethyl acetate–methanol); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.29-2.32 (m, 2H), 2.52-2.54 (m, 2H), 3.33 (br s, 1H),

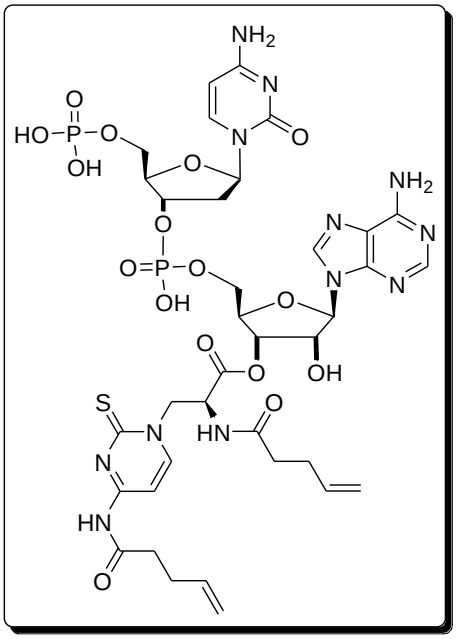
4.96-5.06 (m, 2H), 5.77-5.84 (m, 1H), 7.49 (d, 1H, J = 7.2 Hz), 7.90 (d, 1H, J = 6.8 Hz) and 11.2 (s, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 28.2, 35.4, 99.2, 115.4, 137.0, 147.2, 158.7, 173.4 and 180.4. To a stirred suspension containing 209 mg (1.00 mmol) of compound **3.29** in 5 mL of DMSO was added 0.18 mL (183 mg, 1.20 mmol) of DBU. Within 15 min, a solution containing 220 mg (1.20 mmol) of *N*-(*tert*-Butoxycarbonyl)-L-serine β-lactone in 5 mL of DMSO was added. The reaction mixture was stirred at room temperature for 2 h under argon, then diluted with 10 mL of 0.5 N HCl and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave **3.30** as a colorless solid: yield 265 mg (63%); silica gel TLC R_f 0.15 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.39 (s, 9H), 2.42-2.44 (m, 2H), 2.55-2.58 (m, 2H), 3.74-3.78 (m, 1H), 4.43-4.47 (m, 1H), 4.99-5.11 (m, 3H), 5.87-5.89 (m, 2H), 7.84 (s, 1H) and 8.35 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.6, 28.8, 30.1, 34.2, 37.3, 55.7, 80.5, 106.6, 116.1, 138.0, 158.0, 159.4, 159.5, 172.2 and 172.5; mass spectrum (APCI), m/z 397.1537 $(M+H)^+$ ($C_{17}H_{25}N_4O_5S$ requires m/z 397.1546).



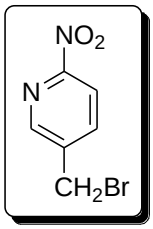
(*S*)-2-pent-4-enamido-3-(4-pent-4-enamido-2-thioxopyrimidin-1(2*H*)-yl)propionic acid (3.31). To a stirred solution containing 127 mg (0.31 mmol) of **3.30** in 2 mL of anhydrous CH_2Cl_2 was added 0.24 mL (353 mg, 3.10 mmol) of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 2 h and then concentrated under diminished pressure. To a stirred solution containing Boc-deprotection product in 2 mL of 1:1 dioxane–water was added 129 mg (0.93 mmol) of K_2CO_3 followed by 61.1 mg (0.31 mmol) of pentenoyl-NHS ester. The reaction mixture was stirred at room temperature for 14 h under argon, then diluted with 20 mL of 0.5 N HCl and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave **3.31** as a light yellow solid: yield 49.8 mg (41% for two steps); silica gel TLC R_f 0.10 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.26-2.56 (m, 8H), 3.51-3.69 (m, 2H), 4.94-5.11 (m, 5H), 5.69-5.85 (m, 5H), 7.40 (br s, 1H), 7.87 (d, 1H, J = 4.4 Hz), 8.34 (d, 1H, J = 4.0 Hz) and 9.16 (br s, 1H); mass spectrum (APCI), m/z 379.1432 $(M+H)^+$ ($C_{17}H_{23}N_4O_4S$ requires m/z 379.1440).



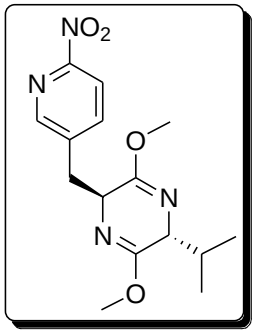
Cyanomethyl (*S*)-2-Pent-4-enamido-3-(4-pent-4-enamido-2-thioxopyrimidin-1(2*H*)-yl)propionate (3.32). To a stirred solution containing 11.8 mg (0.03 mmol) of **3.31** in 1 mL of anhydrous DMF was added 25.0 mg (0.30 mmol) of $NaHCO_3$ followed by 4.0 µL (5.0 mg, 0.06 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for overnight under argon, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave **3.32** as a yellow solid: yield 6.67 mg (53%); silica gel TLC R_f 0.60 (10:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.30-2.34 (m, 4H), 2.48-2.55 (m, 4H), 2.80 (s, 1H), 3.48-3.56 (m, 2H), 4.78 (s, 1H), 5.00-5.09 (m, 4H), 5.74-5.89 (m, 2H), 7.02 (d, 1H, J = 7.2 Hz), 7.88 (d, 1H, J = 6.0 Hz), 8.40 (d, 1H, J = 5.6 Hz) and 8.62 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.9, 29.4, 32.5, 35.6, 36.9, 49.3, 52.5, 106.3, 114.0, 116.0, 116.3, 136.3, 136.6, 157.5, 158.7, 169.6, 169.9, 172.2 and 172.5; mass spectrum (APCI), m/z 418.1550 $(M+H)^+$ ($C_{19}H_{24}N_5O_4S$ requires m/z 418.1549).



((*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(4-amino-2-oxopyrimidin-1(2*H*)-yl)propionic Acid pdCpA Ester (3.5). To a solution containing 5.20 mg (4.00 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 8.76 mg (21.0 μmol) of **3.32**. The reaction mixture was sonicated for 5 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 22.3 min. The fractions containing the product were lyophilized to afford **3.5** as a colorless solid: yield 2.50 mg (65%); mass spectrum (ESI), *m/z* 995.2278 $(M-H)^-$ ($C_{36}H_{45}N_{12}O_{16}P_2$ requires *m/z* 995.2272).

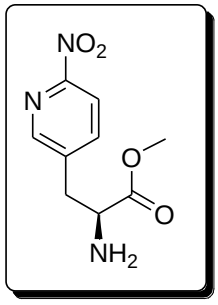


5-(Bromomethyl)-2-nitropyridine (3.33). To a stirred solution containing 250 mg (1.81 mmol) of 5-methyl-2-nitropyridine in tetrachloromethane was added 37.0 mg (0.2 mmol) of 2, 2'-azobisisobutyronitrile and 320 mg (1.8 mmol) of *N*-bromosuccinimide. The reaction mixture was heated at 80 °C for 4 h under argon. The cooled reaction mixture was concentrated under diminished pressure and the residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 3:1 hexanes–ethyl acetate gave the desired product **3.33** as a colorless oil: yield 210 mg (54%); silica gel TLC R_f 0.70 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.55(s, 2H), 8.08 (d, 1H, J = 8.0 Hz), 8.24 (d, 1H, J = 8.5 Hz) and 8.64 (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 27.2, 118.3, 140.1, 140.5 and 149.0; mass spectrum (EI+), m/z 215.9525 $(M)^+$ ($C_6H_5BrN_2O_2$ requires m/z 215.9534).



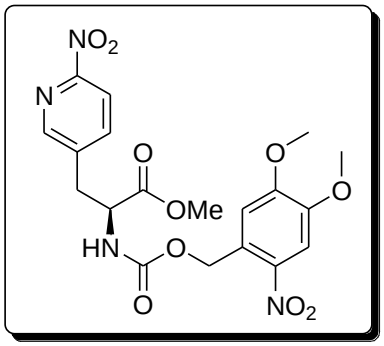
(2*R*,5*S*)-2-Isopropyl-3,6-dimethoxy-5-((6-nitropyridin-3-yl)methyl)-2,5-dihydropyrazine (3.34). To a stirred solution containing 0.17 mL (182 mg, 0.99 mmol) of Schöllkopf's reagent in 3 mL of anhydrous THF at ⁻78 °C was added 0.13 mL (86.1 mg, 1.34 mmol) of 2.5 M BuLi. The reaction mixture was stirred at ⁻78 °C for 30 min under argon and then a solution containing 210 mg (0.98 mmol) of **3.33** in 5 mL of anhydrous THF was added. The reaction mixture was stirred at ⁻78 °C for 5 min under argon, then diluted with 50 mL of satd aq NH_4Cl and extracted with two 50-mL portions

of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 2:1 hexanes–ethyl acetate gave **3.34** as a yellow oil: yield 69.0 mg (22%); silica gel TLC R_f 0.65 (1:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.59 (d, 3H, J = 6.8 Hz), 0.91 (d, 3H, J = 6.8 Hz), 2.10-2.14 (m, 1H), 3.18-3.26 (m, 2H), 3.56-3.57 (m, 1H), 3.62 (s, 3H), 3.68 (s, 3H), 4.26-4.30 (m, 1H), 7.78 (dd, 1H, J = 8.4 and 2.4 Hz), 8.11 (d, 1H, J = 8.4 Hz) and 8.40 (d, 1H, J = 2.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.7, 18.9, 31.9, 36.8, 52.57, 52.63, 55.6, 60.9, 117.3, 140.5, 140.8, 150.2, 155.5, 161.5 and 164.8; mass spectrum (APCI), m/z 321.1563 $(M+H)^+$ ($C_{15}H_{21}N_4O_4$ requires m/z 321.1563).

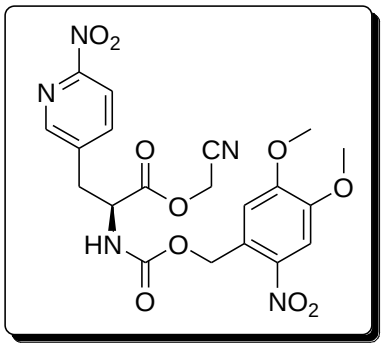


Methyl (*S*)-2-Amino-3-(6-nitropyridin-3-yl)propionate (3.35). To a stirred solution containing 66.0 mg (0.21 mmol) of **3.34** in 4 mL of THF at 0 °C was added 3 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then slowly poured into 50 mL of satd aq $NaHCO_3$ and then extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 4:1 ethyl acetate–methanol gave **3.35** as a yellow oil: yield 39.0 mg (84%); silica gel TLC R_f 0.50 (4:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.02-3.21 (m, 2H), 3.68 (s, 3H), 3.80 (t, 1H, J = 6.8 Hz), 4.80

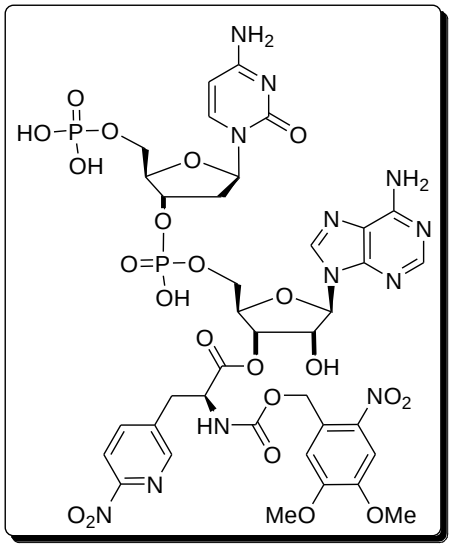
(br s, 2H), 8.04 (dd, 1H, J = 8.4 and 2.0 Hz), 8.23 (d, 1H, J = 8.4 Hz) and 8.45 (d, 1H, J = 2.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 38.2, 52.7, 56.2, 118.9, 142.1, 142.3, 142.4, 150.6 and 175.7; mass spectrum (APCI), m/z 226.0833 $(M+H)^+$ ($C_9H_{12}N_3O_4$ requires m/z 226.0828).



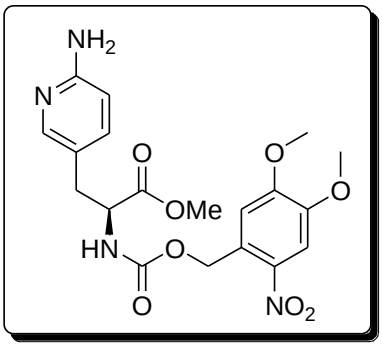
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-nitropyridin-3-yl)propionate (3.36). To a stirred solution containing 30.0 mg (0.13 mmol) of **3.35** in 1 mL of 1:1 dioxane–water was added 55.2 mg (0.39 mmol) of K_2CO_3 followed by 41.3 mg (0.20 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 12 h, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave **3.36** as a yellow solid: yield 33.4 mg (54% for two steps); silica gel TLC R_f 0.65 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.21-3.43 (m, 2H), 3.78 (s, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 4.69-4.73 (m, 1H), 5.48 (s, 2H), 6.95 (s, 1H), 7.70 (s, 1H), 7.85 (d, 1H, J = 8.4 Hz), 8.20-8.22 (m, 1H) and 8.40 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 34.5, 49.5, 54.0, 56.7, 56.9, 63.8, 110.2, 113.6, 117.3, 126.6, 136.9, 140.8, 148.6, 149.0, 153.7, 154.1, 154.3, 156.5 and 169.3; mass spectrum (APCI), *m/z* 465.1261 $(M + H)^+$ ($C_{19}H_{21}N_4O_{10}$ requires *m/z* 465.1258).



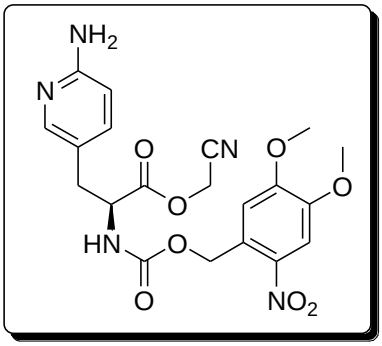
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-nitropyridin-3-yl)propionate (3.37). To a stirred solution containing 46.4 mg (0.10 mmol) of **3.36** in 1 mL of 1:3:1 water–THF–methanol was added 300 µL (0.30 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was dissolved in 1 mL of anhydrous DMF under argon and 50.0 mg (0.60 mmol) of $NaHCO_3$ was added followed by 20.0 µL (25 mg, 0.30 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for overnight under argon, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave **3.37** as a yellow solid: yield 29.0 mg (34%); silica gel TLC R_f 0.67 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.27-3.43 (m, 2H), 3.96 (s, 3H), 3.98 (s, 3H), 4.75-4.89 (m, 3H), 5.46-5.50 (m, 3H), 6.93 (s, 1H), 7.69 (s, 1H), 7.90 (d, 1H, J = 8.4 Hz), 8.23 (d, 1H, J = 8.4 Hz) and 8.45 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 34.9, 49.6, 54.2, 56.6, 56.7, 64.8, 108.5, 111.2, 113.5, 118.3, 126.5, 137.9, 140.8, 148.7, 149.5, 153.6, 154.4, 155.3, 156.2 and 169.5; mass spectrum (APCI), *m/z* 490.1206 $(M + H)^+$ ($C_{20}H_{20}N_5O_{10}$ requires *m/z* 490.1210).



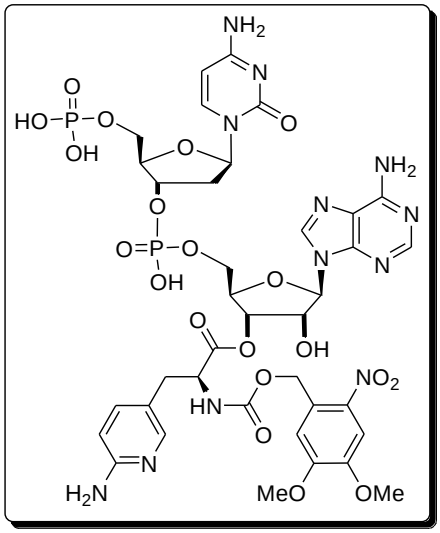
(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-nitropyridin-3-yl)propionic Acid pdCpA Ester (3.6). To a solution containing 5.2 mg (4.0 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 10.3 mg (21 μmol) of **3.37**. The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a C18 reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate (pH 4.5) – acetonitrile. The retention time of the desired product was 24.1 min. The fractions containing the product were lyophilized to afford **3.6** as a colorless solid: yield 2.45 mg (60%); mass spectrum (ESI), *m/z* 1067.1948 $(M-H)^-$ ($C_{37}H_{41}N_{12}O_{22}P_2$ requires *m/z* 1067.1934).



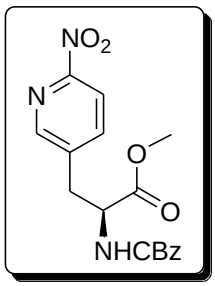
Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-aminopyridin-3-yl)propionate (3.38). To a solution containing 39.0 mg (0.17 mmol) of **3.37** in 5 mL of MeOH was added catalytic amount of 10% Pd/C and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and the filtrate was concentrated under diminished pressure and the residue was redissolved into 1 mL of 1:1 dioxane–water and 62.0 mg (0.45 mmol) of K_2CO_3 was added followed by 55.0 mg (0.20 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for overnight, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:3 methanol–ethyl acetate gave **3.38** as a yellow solid: yield 18.1 mg (23% for two steps); silica gel TLC R_f 0.45 (3:1 methanol–ethyl acetate); ^{1}H NMR (CD_3OD, 400 MHz) δ 2.78-3.05 (m, 2H), 3.71 (s, 3H), 3.89 (s, 3H), 3.91 (s, 3H), 4.36-4.39 (m, 1H), 5.36-5.47 (m, 2H), 6.54 (d, 1H, J = 8.4 Hz), 7.09 (s, 1H), 7.37 (d, 1H, J = 8.4 Hz) and 7.72-7.74 (m, 2H); ^{13}C NMR (CD_3OD, 100 MHz) δ 34.9, 37.1, 52.8, 56.8, 57.0, 64.6, 109.3, 110.4, 110.7, 122.6, 129.7, 140.4, 140.7, 147.8, 149.5, 155.4, 158.1, 159.7 and 173.8; mass spectrum (APCI), *m/z* 435.1512 $(M + H)^+$ ($C_{19}H_{23}N_4O_8$ requires *m/z* 435.1516).



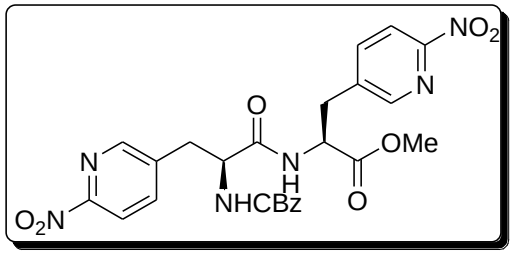
Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-aminopyridin-3-yl)propionate (3.39). To a stirred solution containing 21.8 mg (0.05 mmol) of **3.38** in 1 mL of 1:3:1 water–THF–methanol was added 150 μL (0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was redissolved into 1 mL of anhydrous DMF and 25.0 mg (0.30 mmol) of $NaHCO_3$ was added followed by 10.0 μL (11.3 mg, 0.15 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for overnight under argon, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:3 methanol–ethyl acetate gave the desired product **3.39** as a yellow solid: yield 7.37 mg (32% for two steps); silica gel TLC R_f 0.51 (1:3 methanol–ethyl acetate); ^{1}H NMR (CD_3OD, 400 MHz) δ 2.83-3.06 (m, 2H), 3.90 (s, 3H), 3.92 (s, 3H), 4.46-4.50 (m, 1H), 4.92 (s, 2H), 5.36-5.48 (m, 2H), 6.57 (d, 1H, J = 8.4 Hz), 7.08 (s, 1H), 7.43 (dd, 1H, J = 8.0 and 2.4 Hz), 7.72 (s, 1H), 7.77 (s, 1H) and 7.98 (s, 1H); mass spectrum (APCI), *m/z* 460.1472 $(M + H)^+$ ($C_{19}H_{23}N_4O_8$ requires *m/z* 460.1468).



(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-aminopyridin-3-yl)propionic Acid pdCpA Ester (3.7). To a solution containing 5.20 mg (4.00 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 9.64 mg (21.0 μmol) of cyanomethyl (*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**3.39**). The reaction mixture was sonicated for 5 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 21.3 min. The fractions containing the product were lyophilized to afford **3.7** as a colorless solid: yield 2.21 mg (54%); mass spectrum (ESI), *m/z* 1037.2203 $(M-H)^-$ ($C_{37}H_{43}N_{12}O_{20}P_2$ requires *m/z* 1037.2192).

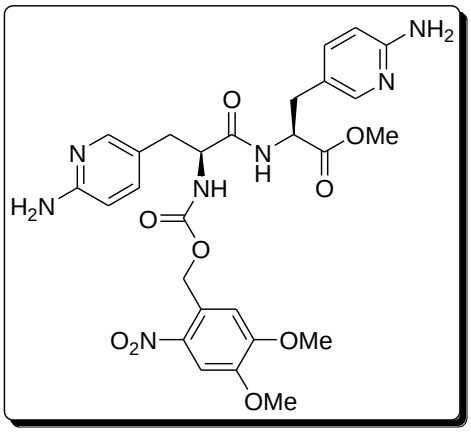


Methyl (*S*)-2-(Benzyloxycarbonylamino)-3-(6-nitropyridin-3-yl)propionate (3.40). To a stirred solution containing 42.8 mg (0.19 mmol) of **3.35** in 1 mL of 1:1 dioxane–water was added 55.2 mg (0.39 mmol) of K_2CO_3 followed by 0.07 mL (32.4 mg, 0.19 mmol) of ClCOOBn. The reaction mixture was stirred at room temperature for 12 h, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave **3.40** as a yellow solid: yield 53.3 mg (78%); silica gel TLC R_f 0.83 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.18-3.21 (m, 1H), 3.35-3.40 (m, 1H), 3.76 (s, 3H), 4.70-4.72 (m, 1H), 5.08 (s, 2H), 5.39 (s, 1H), 7.33-7.37 (m, 5H), 7.77 (d, 1H, J = 8.8 Hz), 8.14 (d, 1H, J = 8.4 Hz) and 8.38 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 35.5, 53.1, 54.4, 67.5, 117.9, 128.4, 128.6, 128.8, 135.9, 138.7, 140.7, 149.6, 155.6, 156.0 and 170.9; mass spectrum (APCI), *m/z* 360.1188 $(M + H)^+$ ($C_{17}H_{18}N_3O_6$ requires *m/z* 360.1196).

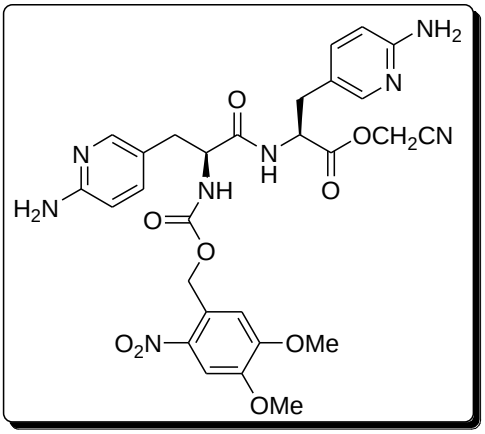


Methyl (*S*)-2-((*S*)-2-(Benzyloxycarbonylamino)-3-(6-nitropyridin-3-yl)propanamido)-3-(6-nitropyridin-3-yl)propionate (3.42). To a stirred solution containing 52.3 mg (0.15 mmol) of **3.40** in 2 mL of 1:3:1 water–THF–methanol was added 300 μL (0.30 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was

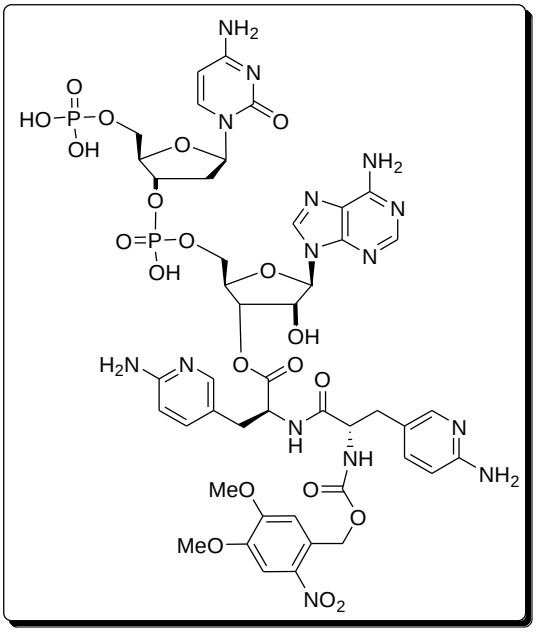
redissolved into 3 mL of anhydrous DMF at 0 °C, 57.2 mg (0.15 mmol) of HBTU was added and resulting solution was stirred at 0 °C for 15 min. To this solution were added 33.8 mg (0.15 mmol) of compound **3.35** and 0.04 mL (30.4 mg, 0.30 mmol) of triethylamine in 1.5 mL of dry DMF. The reaction mixture was stirred at 25 °C for 5 h. The reaction mixture was concentrated under diminished pressure and the residue was diluted with 80 mL of ethyl acetate. The organic layer was washed with two 40-mL portions of 1 N aq HCl, 40 mL of water and 20 mL of brine, then dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 2 cm). Elution with 1:10 methanol−ethyl acetate afforded **3.42** as a colorless oil: yield 42.6 mg (53%); silica gel TLC R_f 0.71 (5:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 3.02-3.33 (m, 4H), 3.72 (s, 3H), 4.53-4.55 (m, 1H), 4.86-5.00 (m, 3H), 5.80-5.82 (m, 1H), 7.09-7.25 (m, 4H), 7.79-7.81 (m, 2H), 8.02-8.12 (m, 2H) and 8.34 (s, 2H); ^{13}C NMR (CD_3OD, 100 MHz) δ 34.7, 35.1, 52.7, 53.1, 55.3, 67.5, 118.0, 128.0, 128.1, 128.6, 128.7, 135.8, 138.9, 139.3, 140.8, 149.4, 149.6, 155.67, 155.74, 155.8, 170.3, 170.5 and 170.7; mass spectrum (APCI), *m/z* 553.1679 $(M + H)^+$ ($C_{25}H_{25}N_6O_9$ requires *m/z* 553.1683).



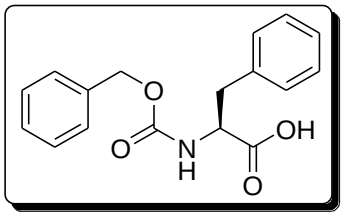
Methyl (*S*)-2-((*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-aminopyridin-3-yl)propanamido)-3-(6-aminopyridin-3-yl)propionate (3.43). To a solution containing 41.0 mg (0.07 mmol) of **3.42** in 5 mL of MeOH was added catalytic amount of 10% Pd/C and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and the filtrate was concentrated under diminished pressure and the residue was redissolved into 1 mL of 1:1 dioxane–water and 20.7 mg (0.15 mmol) of K_2CO_3 was added followed by 19.3 mg (0.07 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 12 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:3 methanol–ethyl acetate gave **3.43** as a yellow solid: yield 10.0 mg (21% for two steps); silica gel TLC R_f 0.50 (1:1 methanol–ethyl acetate); ^{1}H NMR (CD_3OD, 400 MHz) δ 2.84-2.97 (m, 4H), 3.69 (s, 3H), 3.90 (s, 6H), 4.30-4.32 (m, 1H), 4.61 (br s, 1H), 5.41 (br s, 2H), 6.57-6.58 (m, 2H), 7.05-7.06 (m, 1H), 7.36-7.45 (m, 2H) and 7.70-7.75 (m, 3H); mass spectrum (APCI), *m/z* 598.2253 $(M + H)^+$ ($C_{27}H_{32}N_7O_9$ requires *m/z* 598.2261).



Cyanomethyl (*S*)-2-((*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-aminopyridin-3-yl)propanamido)-3-(6-aminopyridin-3-yl)propionate (3.44). To a stirred solution containing 10.2 mg (0.02 mmol) of **3.43** in 1 mL of 1:3:1 water–THF–methanol was added 60 μL (0.06 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was dissolved in 1 mL of anhydrous DMF under argon and 8.33 mg (0.10 mmol) of $NaHCO_3$ was added followed by 4.0 μL (5.0 mg, 0.06 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for overnight under argon, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with 1:3 methanol–ethyl acetate gave **3.44** as a yellow solid: yield 5.20 mg (49% for two steps); silica gel TLC R_f 0.51 (1:1 methanol–ethyl acetate); ^{1}H NMR (CD_3OD, 400 MHz) δ 2.64-3.06 (m, 4H), 3.89 (s, 6H), 4.29-4.30 (m, 1H), 4.66-4.68 (m, 1H), 4.89 (s, 2H), 5.41 (s, 2H), 6.56-6.61 (m, 2H), 7.08 (s, 1H), 7.36-7.48 (m, 2H), 7.73-7.76 (m, 2H) and 7.98 (s, 1H); mass spectrum (APCI), *m/z* 623.2208 $(M + H)^+$ ($C_{28}H_{31}N_8O_9$ requires *m/z* 623.2214).

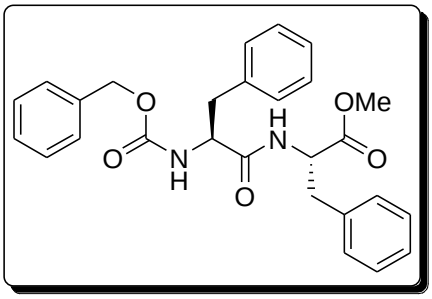


(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-aminopyridin-3-yl)propionic Acid pdCpA Ester (3.8). To a solution containing 5.20 mg (4.00 μmol) of pdCpA tetrabutylammonium salt in 100 μL of 9:1 anhydrous DMF–triethylamine was added 13.1 mg (21.0 μmol) of **3.44**. The reaction mixture was sonicated for 5 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 24.5 min. The fractions containing the product were lyophilized to afford **3.8** as a colorless solid: yield 2.52 mg (55%); mass spectrum (ESI), *m/z* 1200.2963 $(M-H)^-$ ($C_{45}H_{52}N_{15}O_{21}P_2$ requires *m/z* 1200.2937).



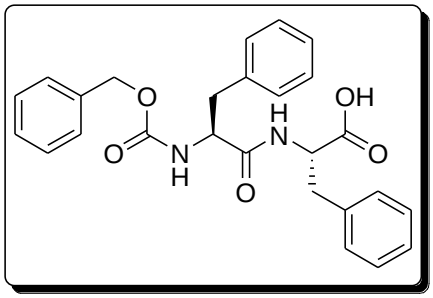
(*S*)-2-(Benzyloxycarbonylamino)-3-phenylpropionic acid (3.45). To a stirred solution containing 500 mg (2.96 mmol) of phenylalanine in 10 mL of 1:1 dioxane–water was

added 510 mg (3.70 mmol) of K_2CO_3 followed by 0.53 mL (631 mg, 3.70 mmol) of ClCOOBn. The reaction mixture was stirred at room temperature for 12 h, then acidified with 10 mL of 1 N aq HCl and the aqueous layer was extracted with three 50-mL portions of ethyl acetate. The combined organic phase was dried over $MgSO_4$ and concentrated under diminished pressure. The crude residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 5:1 ethyl acetate–methanol gave **3.45** as a colorless solid: yield 750 mg (83%); silica gel TLC R_f 0.33 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.15-3.27 (m, 2H), 4.78-4.80 (m, 1H), 5.15 (s, 2H), 5.58-5.60 (m, 1H), 7.21-7.38 (m, 10H) and 10.70 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 37.6, 54.7, 67.2, 127.1, 128.0, 128.2, 128.5, 128.6, 129.3, 135.7, 136.0, 156.1 and 175.8; mass spectrum (APCI), *m*/*z* 300.1233 $(M+H)^+$ ($C_{17}H_{18}NO_4$ requires *m*/*z* 300.1236).



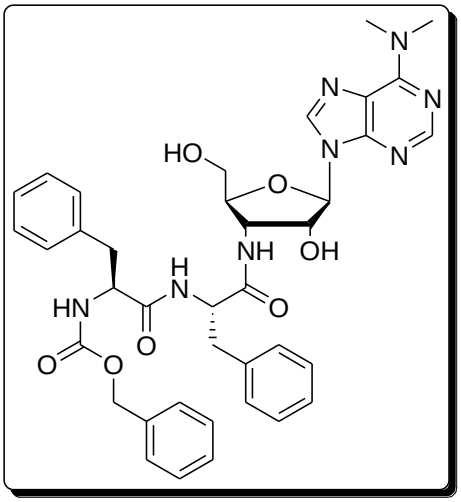
Methyl (*S*)-2-((*S*)-2-(benzyloxycarbonylamino)-3-phenylpropanamido)-3-phenylpropionate (3.46). To a stirred solution containing 993 mg (3.30 mmol) of **3.45** in 5 mL of anhydrous DMF at 0 °C, 1.90 g (4.98 mmol) of HBTU was added and resulting solution was stirred for 15 min. To this solution were added 1.10 g (4.99 mmol) of L-phenylalanine methyl ester and 1.40 mL (1.01 g, 9.98 mmol) of triethylamine in 5 mL of dry DMF. The reaction mixture was stirred at 25 °C for 5 h. The reaction mixture was concentrated under diminished pressure and the residue was diluted with 80 mL of ethyl

acetate. The organic layer was washed with two 40-mL portions of 1 N aq HCl and 20 mL of brine, then dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 4 cm). Elution with ethyl acetate gave **3.46** as a yellow solid: yield 947 mg (62%); silica gel TLC R_f 0.85 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.99-3.11 (m, 4H), 3.63 (s, 3H), 4.60-4.62 (m, 1H), 4.85-4.87 (m, 1H), 4.96-5.09 (m, 2H), 5.79 (d, 1H, J = 8.4 Hz), 6.90 (br s, 1H), 5.58-5.60 (m, 1H) and 7.04-7.38 (m, 14H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 37.9, 38.4, 52.1, 53.3, 55.9, 66.8, 126.8, 126.9, 127.8, 128.0, 128.4, 129.2, 129.3, 135.7, 136.4, 155.88, 155.90, 170.8 and 171.4; mass spectrum (APCI), *m*/*z* 461.2065 $(M+H)^+$ ($C_{27}H_{29}N_2O_5$ requires *m*/*z* 461.2076).



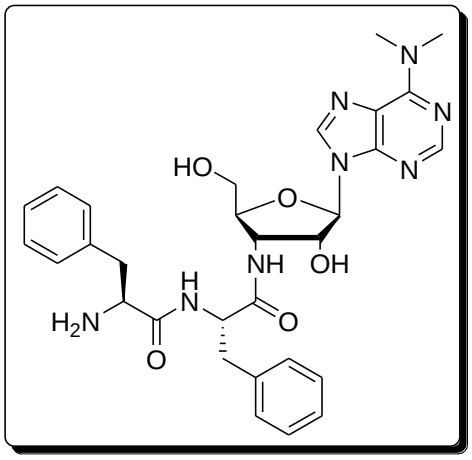
(*S*)-2-((*S*)-2-(Benzyloxycarbonylamino)-3-phenylpropanamido)-3-phenylpropionic acid (3.47). To a stirred solution containing 715 mg (1.55 mmol) of **3.46** in 5 mL of 1:3:1 water–THF–methanol was added 111 mg (4.65 mmol) of LiOH in 3 mL of water. The reaction mixture was stirred at room temperature for 2 h, and then then acidified with 1 N aq HCl and the aqueous layer was extracted with three 50-mL portions of ethyl acetate. The combined organic extract was dried ($MgSO_4$) and concentrated under diminished pressure to afford the crude product. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 5:1 ethyl acetate–methanol gave **3.47** as a colorless solid: yield 590 mg (85%); silica gel TLC R_f 0.75 (1:1 ethyl acetate–methanol);

^{1}H NMR (CD_3OD, 400 MHz) δ 2.68-3.17 (m, 4H), 4.45-4.71 (m, 2H), 4.87-5.10 (m, 4H) and 7.17-7.22 (m, 15H); ^{13}C NMR (CD_3OD, 100 MHz) δ 38.4, 38.9, 54.5, 57.5, 67.5, 127.7, 128.5, 128.8, 129.3, 129.4, 130.2, 130.3, 130.4, 137.9, 138.4, 157.9, 173.8 and 174.2; mass spectrum (APCI), *m*/*z* 447.1919 $(M+H)^+$ ($C_{27}H_{27}N_2O_5$ requires *m*/*z* 447.1920).



9-[3′-Deoxy-3′-((*S*)-2-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-3-phenylpropanoyl)-β-D-ribofuranosyl]-6-(N, N-dimethylamino)purine (3.48). To a stirred solution containing 89.2 mg (0.20 mmol) of **3.47** and 35.0 mg (0.30 mmol) of *N*-hydroxysuccinimide in 3 mL of anhydrous CH_2Cl_2 at 0 °C was added dropwise 61.0 mg (0.30 mmol) of DCC dissolved in 3 mL of anhydrous CH_2Cl_2. The reaction mixture was stirred at room temperature for 18 h, and then concentrated under diminished pressure and suspended in CH_3CN to permit the removal of dicyclohexylurea. The suspension was filtered and the filtrate was concentrated under diminished pressure. The resulting solid was used in the next reaction without further purification. To a stirred solution containing 130 mg (0.24 mmol) of this crude intermediate and 47.0 mg (0.16 mmol) of puromycin aminonucleoside in 1.5 mL of dry DMF was added 0.30 mL (24 mg, 0.24 mmol) of Et_3N.

The reaction mixture was stirred at 25 °C for 3 h, and then concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave **3.48** as a yellow solid: yield 36.0 mg (25%); silica gel TLC R_f 0.31 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.73-2.78 (m, 2H), 2.96-3.10 (m, 4H), 3.45 (s, 6H), 3.46-3.47 (m, 1H), 3.80-3.82 (m, 2H), 3.99 (br s, 1H), 4.32-4.35 (m, 1H), 4.54-4.58 (m, 3H), 4.65-4.67 (m, 1H), 4.99-5.01 (m, 2H), 5.93 (br s, 1H), 7.12-7.27 (m, 15H), 8.16 (br s, 1H) and 8.31 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 26.0, 26.7, 34.7, 38.9, 51.8, 56.0, 57.9, 62.1, 67.7, 75.0, 84.6, 91.9, 121.6, 127.7, 128.6, 129.4, 129.5, 130.2, 130.4, 137.8, 138.0, 138.1, 139.0, 150.4, 152.8, 156.0, 173.3 and 173.9; mass spectrum (APCI), *m*/*z* 723.3265 $(M+H)^+$ ($C_{38}H_{43}N_8O_7$ requires *m*/*z* 723.3255).



9-[3′-Deoxy-3′-((*S*)-2-((*S*)-2-amino-3-phenylpropanamido)-3-phenylpropanoyl)-β-D-ribofuranosyl]-6-(N, N-dimethylamino)purine (3.9). To a solution containing 36.0 mg (0.05 mmol) of **3.48** in 3 mL of MeOH was added catalytic amount of 10% Pd/C and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and the filtrate was concentrated under diminished

pressure and purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave **3.9** as a colorless solid: yield 98.0 mg (47%); silica gel TLC R_f 0.51 (1:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.71-2.75 (m, 2H), 2.99-3.07 (m, 4H), 3.48 (s, 6H), 3.52-3.55 (m, 2H), 3.67-3.70 (m, 2H), 3.77-3.81 (m, 1H), 3.93-3.95 (m, 2H), 4.55-4.56 (m, 2H), 4.68-4.72 (m, 1H), 5.93 (br s, 1H), 7.17-7.30 (m, 10H), 8.18 (br s, 1H) and 8.33 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 39.3, 41.3, 52.0, 56.1, 56.9, 62.3, 75.1, 84.8, 92.0, 121.6, 127.99, 128.00, 129.58, 129.62, 129.65, 129.69, 129.71, 130.3, 130.38, 130.43, 130.5, 138.0, 138.1, 139.2, 150.6, 153.0, 156.1, 173.5 and 173.9; mass spectrum (APCI), *m*/*z* 589.2898 $(M+H)^+$ ($C_{30}H_{37}N_8O_5$ requires *m*/*z* 589.2887).

CHAPTER 4

SYNTHESIS OF *N*-HYDROXYPYRIDONES AS MULTIFUNCTIONAL RADICAL QUENCHERS

4.1. Introduction

Coenzyme Q_{10} (CoQ_{10}) is one of the electron carriers in the electron transport chain, mediating transport from Complexes I and II to ubiquinone-cytochrome *c* reductase (Complex III). Its reduced form, $CoQ_{10}H_2$ is one of the most potent lipophilic antioxidants in cell membranes as it inhibits lipid peroxidation *in vitro* by quenching lipid radicals and superoxide.[104-107,170-172] The reduced state, $CoQ_{10}H_2$ can be converted back to CoQ_{10} by the respiratory chain via redox cycling (Figure 4.1).[170-172] CoQ_{10} has been tested for the treatment of illnesses related to mitochondrial dysfunction like Friedreich's ataxia but its low solubility (octanol-water partition coefficient > 10^{20}) makes it poorly bioavailable so that it cannot easily gain access to the mitochondria. Without access to the mitochondrial inner membrane of mitochondria, exogenous CoQ_{10} is unable to restore normal respiration.[170,173]

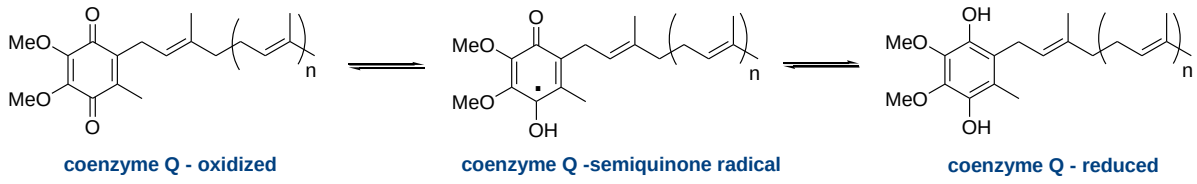


Figure 4.1. General scheme showing redox cycling of coenzyme Q_n where n is the number of isoprenoid Units.

There has been growing interest in the development of CoQ_{10} analogues having modified side chains in order to impart good pharmacokinetic properties and

mitochondrial membrane absorption. Recently, idebenone was proposed as an analogue of CoQ_{10} capable of functioning as a neuroprotective agent. Idebenone has been shown to accept electrons from mitochondrial Complex I and to restore respiration in ubiquinone-deficient mitochondria. Idebenone has a shorter aliphatic chain than CoQ_{10}, which makes it less lipophilic, but it is still lipophilic enough to interact with lipid bilayers. The compound has been the subject of a number of clinical trials for the treatment of inherited disorders of the respiratory chain;[174-177] however, a few studies have shown that idebenone fails to increase ATP levels in CoQ_{10} deficient fibroblasts at high doses and also inhibits Complex I in the respiratory chain.[178-180]

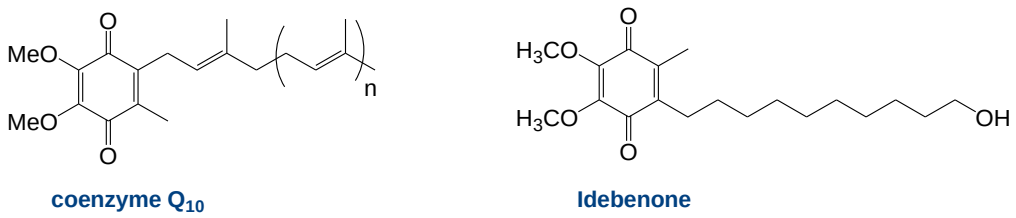


Figure 4.2. Structures of natural and synthetic antioxidants CoQ_{10} and idebenone.

The natural electron carrier, α-tocopherol (α-TOH) is one of the best lipophilic antioxidants.[109,110] The activity of phenolic antioxidants in quenching lipid radicals depends on their ability to transfer the phenolic hydrogen atom to a carbon or peroxyl radical, as well as on the stability of the formed phenoxyl radical. It has been found that an increase in ring electron density of phenolic antioxidants lowers the O-H bond dissociation energy (O-H BDE) by stabilizing the phenoxyl radical, thus their activity as antioxidants is enhanced. However, some antioxidants with strong electron-donating substituents are less stable to air oxidation.[181-183] Air stability is correlated to the

ionization potential (IP) of molecules: with a lower IP value, it is easier for a molecule to lose electrons and be oxidized.[181-183] An antioxidant having a very low IP, could transfer an electron to molecular oxygen, and may become a source of superoxide rather than acting as antioxidant. To improve the air stability of such molecules, a series of nitrogen heterocyclic α-TOH type analogues (Figure 4.3) having one nitrogen atom (3-position) or two nitrogen atoms (3- and 5- positions) in the phenolic ring have been studied.[181-183] The incorporation of a nitrogen atom in the phenolic ring increased the ionization potential (IP) and thus improved the air stability.

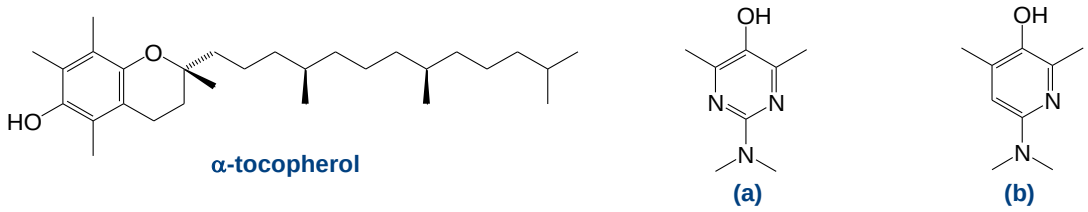


Figure 4.3. Chemical structures of α-TOH type analogues having pyrimidine (a) and pyridine (b) redox cores.

The pyrimidinol and pyridinol analogues have been found to exhibit favorable antioxidant properties. They scavenge free radicals, inhibit lipid peroxidation and preserve mitochondrial function from oxidative stress in a number of cell lines. Some of the pyrimidinol analogues augment ATP production in cultured CoQ_{10} deficient lymphocytes and FRDA fibroblasts and lymphocytes.[184-187] These compounds have been termed multifunctional radical quenchers (MRQs), having multiple functions in the cell such as accepting electrons from superoxide, quenching carbon-centered lipid radicals and restoring ATP production in ATP-deficient cells.[186,187] These encouraging results prompted us to explore other aza analogues of α-TOH or idebenone that might lead to more potent and efficacious compounds. *N*-hydroxy-4-pyridones (Figure 4.4) bear

structural resemblance to the quinone moiety of CoQ_{10}. Their particular structure should allow them to stabilize radicals more effectively than quinones. The presence of a nitrogen atom in the ring increases the ionization potential, which is expected to stabilize the presence of strong electron donating groups without affecting the bond dissociation energy between oxygen and hydrogen. Therefore, these molecules should behave as quenchers of reactive oxygen species and the presence of a more hydrophilic core should afford better solubility in aqueous environments. In order to test this thesis, a series of analogues containing a *N*-hydroxy-4-pyridone core has been prepared. To enable a study of their ability to reach mitochondria and their interaction with the electron transport chain, different lipophilic side chains were employed (Figure 4.4). A hypothetical catalytic cycle for the *N*-hydroxy-4-pyridones acting as lipid radical and superoxide quenchers is outlined in Figure 4.5. Quenching of lipid peroxidation by *N*-hydroxy-4-pyridone would result in a radical that should be a strong electron acceptor. This species should be capable of reacting with superoxide, the form of ROS that is expected to be produced in partially dysfunctional mitochondria through the leakage of electrons from the electron transport chain onto oxygen.

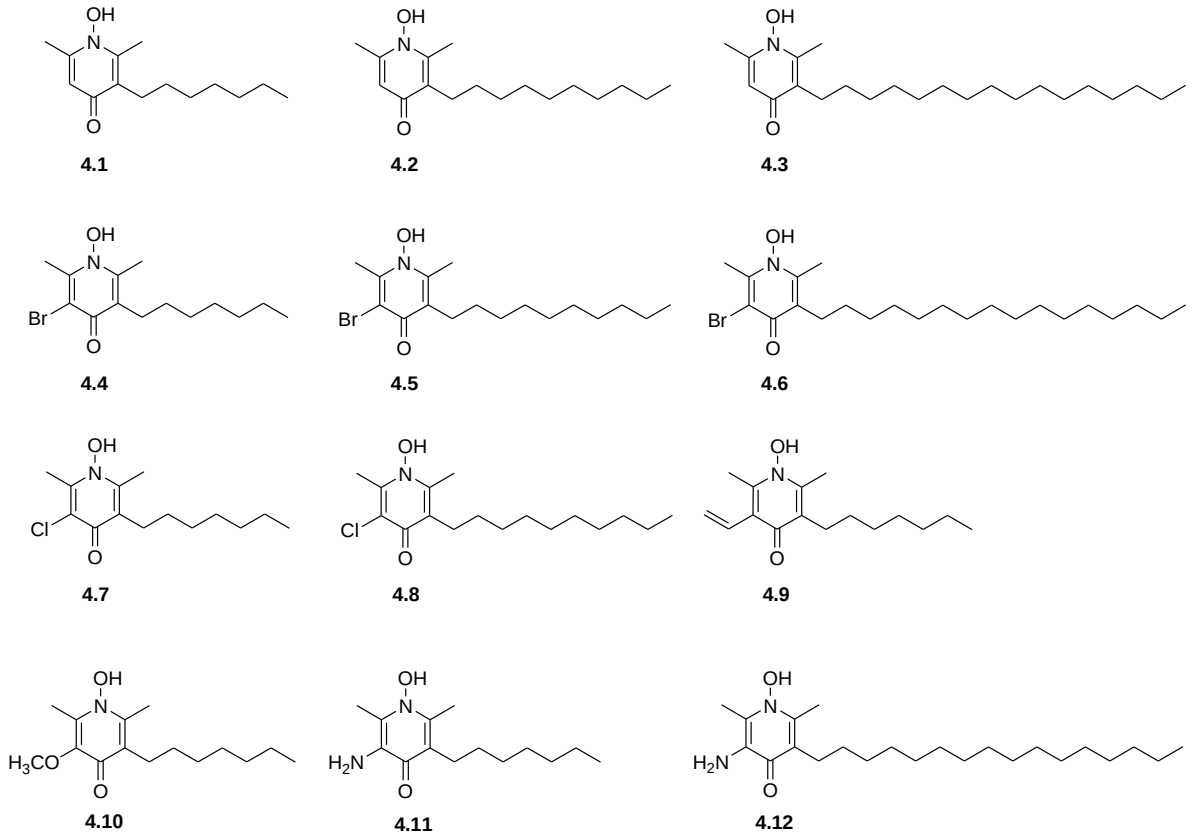


Figure 4.4. *N*-Hydroxypyridone analogues synthesized and evaluated.

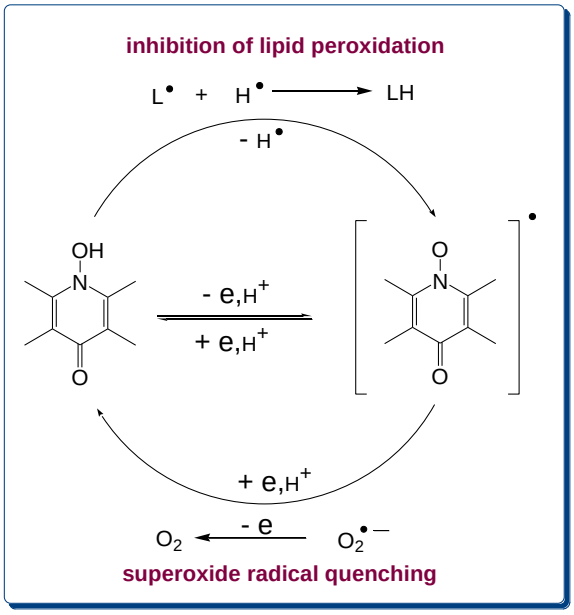


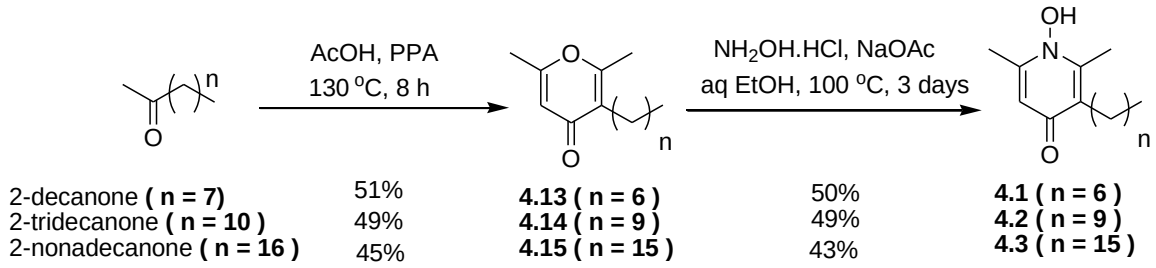
Figure 4.5. Proposed catalytic cycle for the *N*-hydroxypyridones acting as lipid radical and superoxide quencher (adapted from ref 184).

4.2. Results

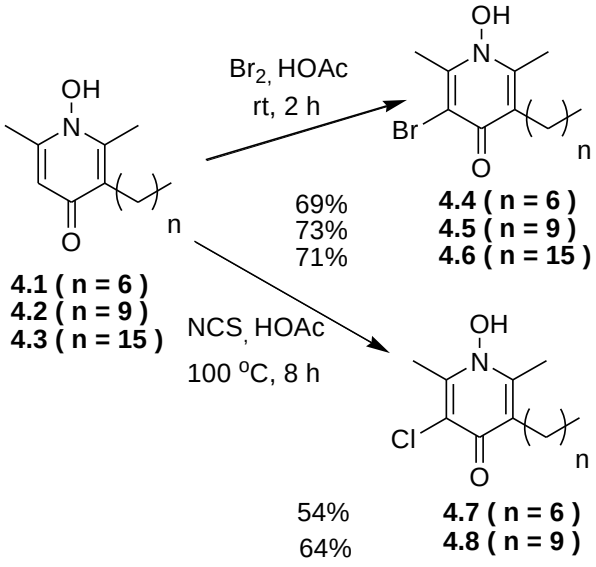
Synthesis of *N*-Hydroxy-4-pyridones

The aim of this study was to synthesize and evaluate as MRQs a library of *N*-hydroxy-4-pyridones having long alkyl chains. The commercially available long chain methyl ketones, 2-decanone, 2-tridecanone and 2-nonadecanone, were treated with polyphosphoric acid (PPA) and acetic acid to produce trimethyl-γ-pyrones (**4.13**, **4.14** and **4.15**, respectively) in 45%-51% yields (Scheme 4.1).[188] Treating these trimethyl-γ-pyrones with hydroxylamine led to the formation of *N*-hydroxypyridones **4.1**, **4.2** and **4.3**, respectively, in 43%-50% yields. Compounds **4.1**, **4.2** and **4.3** were brominated by treatment with Br_2 in acetic acid to afford **4.4**, **4.5** and **4.6**, respectively, in 69%-73% yields (Scheme 4.2). Chlorination of the compounds **4.1** and **4.2** with *N*-chlorosuccinamide (NCS) in acetic acid produced compounds **4.7** and **4.8** in 54% and 64% yields, respectively (Scheme 4.2).[189]

Trimethyl-γ-pyrone **4.13** was treated with ammonium hydroxide to produce NH-pyridone **4.16** in 51% yield (Scheme 4.3). Iodination of the pyridone **4.16** in presence of ceric ammonium nitrate (CAN) afforded the iodopyridone **4.17** in 68% yield.[190] Treatment of the iodopyridone with Boc anhydride produced the Boc-protected compound **4.18** in 75% yield.[191] Compound **4.18** underwent tetrakis(triphenylphosphine)palladium-mediated Stille coupling with tributyl(vinyl)tin to produce compound **4.19** in 27% yield.[192] N-oxidation using mCPBA converted the compound **4.19** to N-oxide **4.20**.[191] Boc deprotection of compound **4.20** using 10 M KOH afforded the desired vinyl-substituted *N*-hydroxypyridone **4.9** (Scheme 4.3).[191]

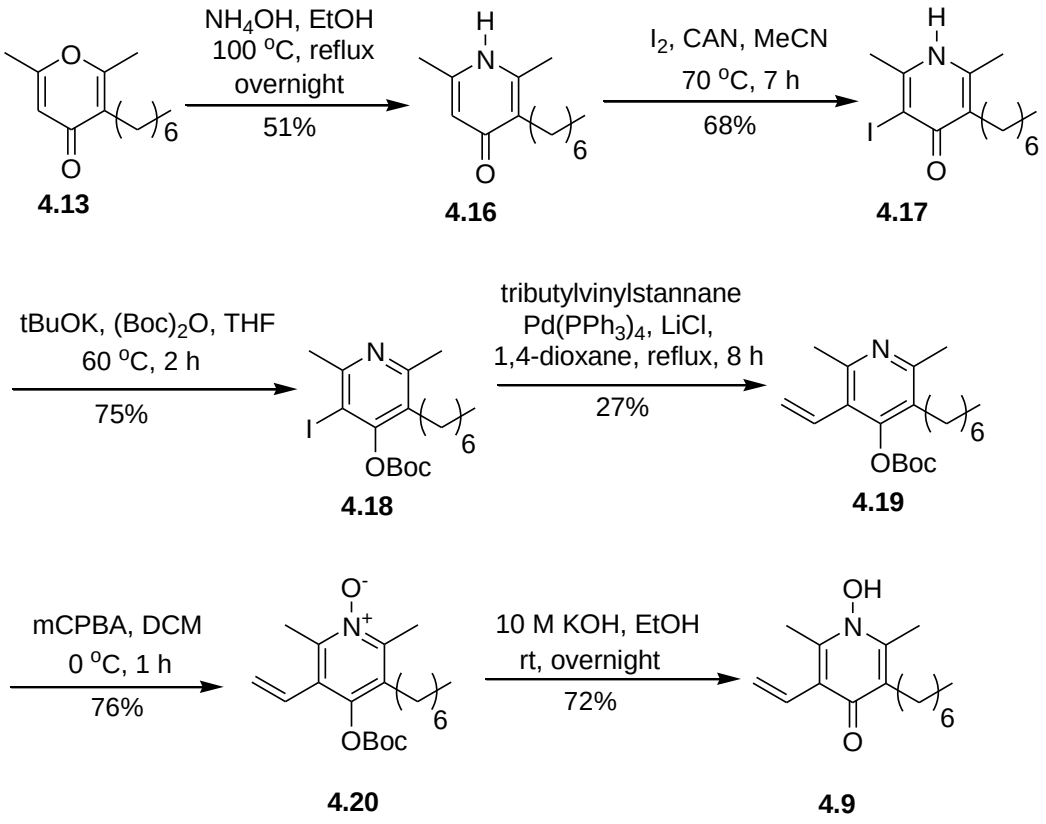


Scheme 4.1. Synthesis of *N*-Hydroxypyridone Analogues Having Linear Alkyl Side Chains.

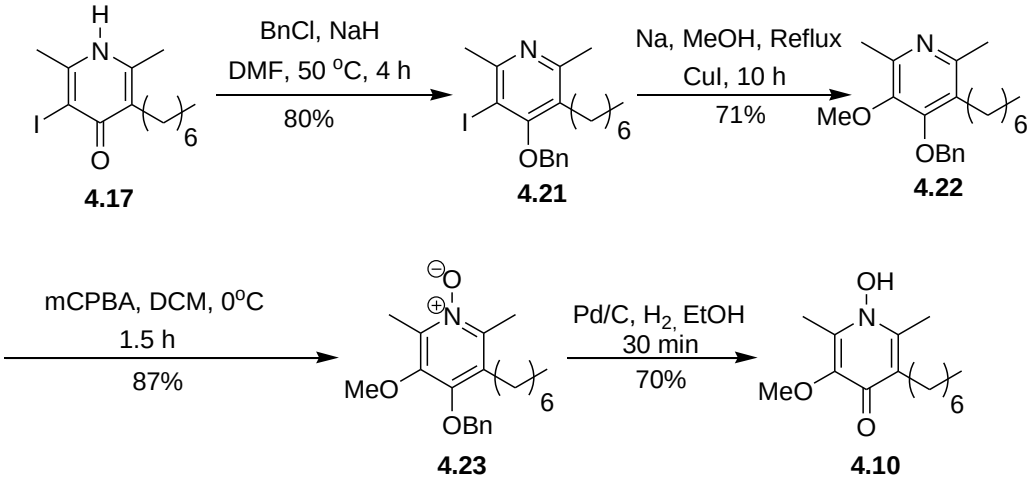


Scheme 4.2. Synthesis of Bromo- and Chloro-substituted *N*-Hydroxy-4-pyridones.

Iodo derivative **4.17** was treated with benzyl chloride (BnCl) and sodium hydride (NaH) to afford OBn-protected compound **4.21** in 80% yield (Scheme 4.4).[193] Treatment of **4.21** with metallic sodium in methanol formed **4.22** in 71% yield.[189] *m*-Chloroperoxybenzoic acid (mCPBA) converted **4.22** to the N-oxide **4.23** in 87% yield.[191] Hydrogenolysis over 10% Pd/C produced the desired methoxy-substituted pyridone **4.10** in 70% yield (Scheme 4.4).[194]

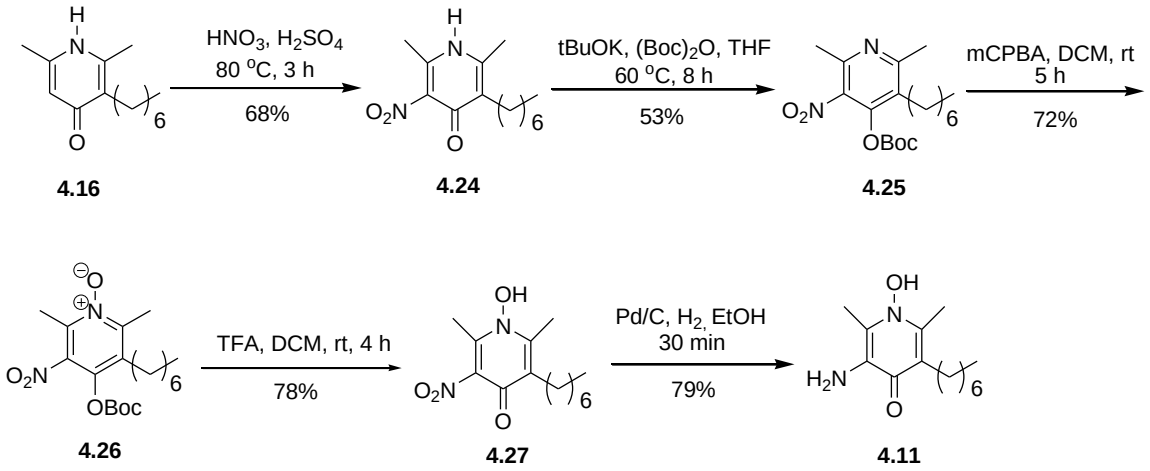


Scheme 4.3. Synthesis of Vinyl-substituted *N*-Hydroxy-4-pyridone **4.9**.



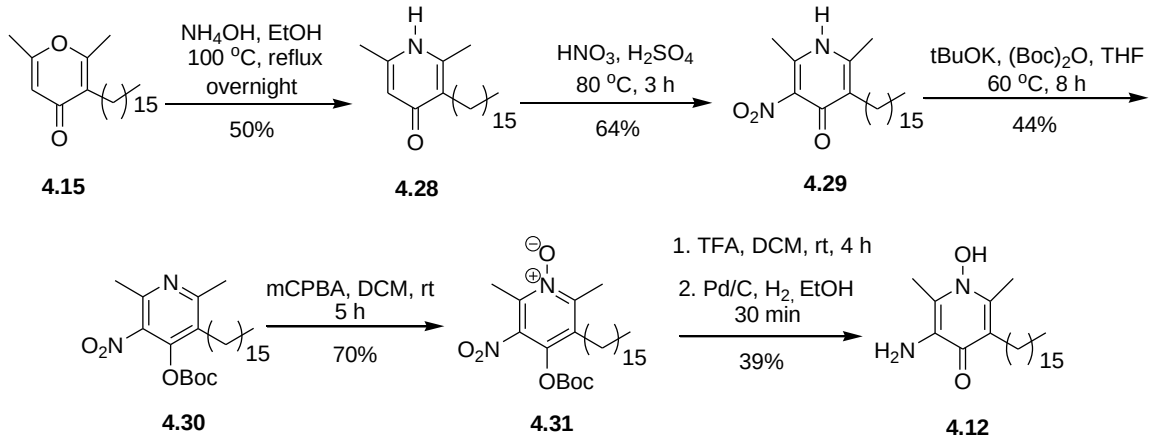
Scheme 4.4. Synthesis of Methboxy-substituted *N*-Hydroxy-4-pyridone **4.10**.

NH-pyridone **4.16** (Scheme 4.5) was treated with a HNO_3/H_2SO_4 mixture to produce nitro pyridone **4.24** in 68% yield.[195] Treatment of nitropyridone **4.24** with Boc anhydride produced **4.25** in 53% yield. N-oxidation using mCPBA converted **4.25** to the N-oxide **4.26** in 72% yield. Boc deprotection of N-oxide **4.26** by trifluoroacetic acid (TFA) produced the nitro pyridone **4.27** which underwent reduction of the nitro group over Pd/C to form the desired amino-substituted *N*-hydroxy-4-pyridone **4.11** in 79% yield (Scheme 4.5).



Scheme 4.5. Synthesis of Amino-substituted *N*-Hydroxy-4-pyridone **4.11**.

Trimethyl-γ-pyrone **4.15** was treated with ammonium hydroxide to produce NH-pyridone **4.28** in 50% yield (Scheme 4.6). NH-pyridone **4.28** was treated with a HNO_3/H_2SO_4 mixture to produce the compound **4.29** in 64% yield. Treatment of nitropyridone **4.29** with Boc anhydride produced **4.30** in 44% yield. N-oxidation using mCPBA converted **4.30** to the respective N-oxide **4.31** in 70% yield. Boc deprotection of the compound **4.31** by TFA, followed by reduction of the nitro group afforded the desired amino-substituted *N*-hydroxy-4-pyridone **4.12** in 39% overall yield (Scheme 4.6).



Scheme 4.6. Synthesis of Amino-substituted *N*-Hydroxy-4-pyridone **4.12**.

Biological Evaluation of the *N*-Hydroxy-4-pyridones

In order to evaluate the structural effects of analogues **4.1**-**4.12**, the compounds were tested for NADH oxidase activity, suppression of lipid peroxidation, ROS scavenging and in cell viability assays.

Mitochondrial Electron Transport Chain Function (NADH Oxidase Activity)

Inhibition of any of the mitochondrial electron transport chain complexes can limit the potential therapeutic use of MRQ analogues. It is important to prepare compounds lacking any inhibitory effect on the respiratory chain. Any ETC inhibition will ultimately reduce mitochondrial ATP production. Since, ETC begins with the oxidation of NADH and channeling the derived electrons into the formation of reduced coenzymes, ETC function can be estimated through a change in the rate of NADH oxidation. The NADH oxidase assay evaluates the inhibitory effect of molecules on the ETC. Molecules having therapeutic potential should exhibit only limited inhibition of NADH oxidase activity even at higher concentrations.

The inhibitory effects of *N*-hydroxy-4-pyridone analogues were evaluated using submitochondrial particles (SMP). The results (performed by Dr. Sriloy Dey) presented in Tables 4.1 and Table 4.2 show that compound **4.3** (Table 4.1), having a 16-carbon atom side chain was less inhibitory than the corresponding analogues having 10 or 7-carbon atom side chains. Compound **4.9**, having a 7-carbon atom side chain and a vinyl group, and compound **4.12**, having a 16 carbon atom side chain and an amino group, were the most inhibitory analogues (Table 4.2).

Table 4.1. NADH Oxidase Activity of *N*-Hydroxypyridone Analogues (**4.1-4.8**) are Shown Relative to Untreated Control. Data are expressed as the mean ± SEM (n = 3).

	NADH oxidase (Complex I, III, IV) activity (%)	
Compound	0.5 μM	1 μM
untreated control	100	100
4.1	70 ± 2	67 ± 1
4.3	85 ± 3	79 ± 2
4.4	60 ± 3	55 ± 1
4.5	44 ± 1	42 ± 1
4.7	64 ± 1	61 ± 2
4.8	79 ± 2	75 ± 1

Table 4.2. NADH Oxidase Activity of *N*-Hydroxypyridone Analogues (**4.9-4.12**) are Shown Relative to Untreated Control. Data are expressed as the mean ± SEM (n = 3).

Compound	NADH oxidase (Complex I, III, IV) activity (%)		
	1 μM	5 μM	10 μM
untreated control	100	100	100
4.9	29.7 ± 3.8	20.7 ± 2.2	13.2 ± 1
4.10	74.5 ± 6.9	44.5 ± 3.6	33.5 ± 2.2
4.11	92.9 ± 10.9	76.7 ± 6.9	70.4 ± 5.5
4.12	17.7 ± 1	12.7 ± 1.7	10.7 ± 1.3

Inhibition of Lipid Peroxidation

The ability of the synthesized analogues to quench lipid peroxidation was evaluated in cultured CEM leukemia lymphocytes. These cells were placed under oxidative stress by depleting them of glutathione (GSH) by treatment with diethyl maleate (DEM). The extent of lipid peroxidation was quantified using a hydrophobic fatty acid fluorophore C_{11}-BODIPY$^{581/591}$, which inserts preferentially in membranes. Upon oxidation of the polyunsaturated butadienyl portion of the fluorophore, the red emitting form of the dye (λ_{em} 595 nm) is converted into a green emitting form (λ_{em} 520 nm). Increased C_{11}-BODIPY$^{581/591}$-green fluorescence, a measure of peroxyl radical production, was quantified by flow cytometric analysis, which is expressed as percent scavenging activity.

The results in Table 4.3 show that most of the analogues were inactive or poorly effective in suppressing lipid peroxidation. Compound **4.3** (Table 4.3) had some lipid peroxidation suppressing ability, whereas the corresponding analogues **4.1** and **4.2** were

inactive, suggesting that alkyl side chain length was important. Amino-substituted *N*-hydroxypyridone analogue **4.12** was best analogue in suppressing lipid peroxidation at 5 μM concentration (94% suppression of lipid peroxidation).

Table 4.3. Suppression of Lipid Peroxidation by *N*-Hydroxypyridone Analogues (**4.1-4.12**) in Cultured CEM Lymphocytes Treated with DEM. The experiment was performed by Dr. Omar M. Khdour.

Compound	Scavenging activity (%)	
	1 μM	5 μM
untreated control	100	100
treated control	0	0
4.1	0	0
4.2	0	0
4.3	10 ± 4.3	20 ± 3.4
4.4	0	0
4.5	0	0
4.6	7 ± 2.3	15 ± 3.3
4.7	0	0
4.8	0	0
4.9	0	0
4.10	11 ± 1.8	2.2 ± 2.3
4.11	14 ± 4.1	9.6 ± 2.8
4.12	18 ± 2.2	94 ± 0.2

Suppression of Reactive Oxygen Species

Some of the *N*-hydroxy-4-pyridone analogues **4.9**-**4.12** were assayed for suppression of reactive oxygen species (ROS) production in cultured CEM leukemia cells pretreated with DEM. The intracellular ROS level was measured based on the ROS-induced formation of the highly fluorescent product 2′,7′-dichlorofluorescein (DCF) from the non-fluorescent dye 2′,7′- dichlorodihydrofluorescein diacetate (DCFH-DA). The results presented in Table 4.4 show **4.11** and **4.12** exhibited dose-dependent behaviors in suppressing ROS. Amino-substituted *N*-hydroxypyridone **4.12** having a 16-carbon atom substituent was the most effective in suppressing ROS, in agreement with the present lipid peroxidation results.

Table 4.4. Suppression of ROS Production in Cultured CEM Leukemia Cells Pretreated with DEM. Results are Expressed as % Scavenging Activity. The experiment was performed by Dr. Ruth Goldschmidt.

Compound	ROS Scavenging activity (%)		
	0.25 μM	2.5 μM	10 μM
untreated control	100	100	100
treated control	0	0	0
4.9	23.4 ± 1.6	22.2 ± 3.5	20.0 ± 4.4
4.10	21.0 ± 5.5	26.4 ± 4.4	0 ± 10
4.11	4.6 ± 5.4	42.2 ± 2.9	67.0 ± 5.9
4.12	41.4 ± 14.9	81.8 ± 3.7	91.0 ± 1.5

Cytoprotection

The ability of the test compounds (**4.1**–**4.12**) to confer cytoprotection to cultured cells under DEM-induced oxidative stress was evaluated (Table 4.5). Cell viability was determined by trypan blue exclusion assay in an FRDA cell line. This technique was used to assess the cytoprotective effects of the compounds in cultured cells treated with diethyl

Table 4.5. Cytoprotection of Cultured FRDA Lymphocytes from the Effects of DEM Induced Oxidative Stress. Results are Expressed as % Viability. The experiment was performed by Dr. Ruth Goldschmidt.

	% Viable Cells				
Compound	0.1 μM	0.5 μM	2.5 μM	untreated	Treated
4.1	27 ± 7	30 ± 13	24 ± 6	93 ± 7	21 ± 8
4.2	36 ± 6	14 ± 5	20 ± 3	97 ± 9	17 ± 2
4.3	19 ± 6	15 ± 5	18 ± 2	97 ± 9	17 ± 2
4.4	31 ± 12	28 ± 7	32 ± 5	97 ± 5	18 ± 3
4.5	19 ± 6	20 ± 13	32 ± 8	97 ± 5	18 ± 3
4.6	12 ± 5	6 ± 3	8 ± 3	96 ± 2	12 ± 3
4.7	15 ± 6	29 ± 3	39 ± 3	97 ± 5	18 ± 3
4.8	17 ± 3	14 ± 2	19 ± 5	97 ± 9	17 ± 2
4.9	25 ± 6	42 ± 3	30 ± 6	93 ± 7	21 ± 8
4.10	12 ± 6	17 ± 7	14 ± 3	96 ± 2	12 ± 3
4.11	19 ± 6	19 ± 3	25 ± 5	96 ± 2	12 ± 3
4.12	17 ± 5	21 ± 9	35 ± 9	96 ± 2	12 ± 3

maleate to induce cell death by depletion of cellular glutathione. The viability of DEM-treated FRDA cells was determined by their ability to exclude the dye trypan blue. None of the compounds provided significant cytoprotection from DEM-induced stress.

4.3. Discussion

There has been no study to date of the efficiency of *N*-hydroxy-4-pyridones analogues as radical quenchers. In this study, some analogues have been synthesized that can provide data relevant to the structural requirements for mitochondrial protection. Trimethyl-γ-pyrones (**4.13**, **4.14** and **4.15**) were prepared from commercially available long chain methyl ketones.[188] These trimethyl-γ-pyrones produced *N*-hydroxy-4-pyridones (**4.1**, **4.2** and **4.3**) under reflux condition (Scheme 4.1), whereas the synthesis of NH-pyridones (**4.16** and **4.28**) was performed in a high pressure tube (Schemes 4.3 and 4.6). Both the syntheses of *N*-OH pyridones and *N*-H pyridones from pyrones were low yielding, needing longer reaction times. The reactivity of the pyrones decreased with an increasing number of carbon atoms in the side chains, making the ring more electron rich and less likely to be attacked by nucleophiles such as hydroxylamine or ammonia (Schemes 4.1, 4.2 and 4.6). *N*-hydroxy-4-pyridones (**4.1**, **4.2** and **4.3**) underwent electrophilic bromination or chlorination to afford bromo-and chloro-substituted *N*-hydroxy-4-pyridones (**4.4**-**4.8**).

Vinyl-, methoxy- and amino-substitued *N*-hydroxy-4-pyridones **4.9**-**4.12** (Schemes 4.3, 4.4, 4.5 and 4.6) were synthesized anticipating that these substitutions would stabilize the pyridonoxyl radical more effectively. The synthesis of vinyl-substitued pyridone **4.9** commenced with iodination of NH-pyridone **4.16**. Boc protection

of NH-pyridone **4.17** at 60 ^{o}C afforded **4.18**. Compound **4.18** underwent tetrakis(triphenylphosphine)palladium mediated Stille coupling with tributyl(vinyl)tin to afford **4.19**. N-oxidation using mCPBA, followed by Boc deprotection using 10 M KOH, afforded the desired vinyl-substituted *N*-hydroxypyridone **4.9** (Scheme 4.3).

Benzylation of iodopyridone **4.17** was effected using benzyl chloride at 50 ^{o}C in order to obtain the thermodynamically stable *O*-benzylated product rather than the *N*-benzylated product (Scheme 4.4). N-Oxidation using mCPBA in dichloromethane, and the removal of the benzyl protecting group by hydrogenolysis, afforded the desired *N*-OH pyridone **4.10** (Scheme 4.4). Nitration of the pyridones had to be done using a mixture of HNO_3 and H_2SO_4 at high temperature. The Boc protection reaction of nitro pyridones (**4.24** and **4.29**) and the subsequent N-oxidation reactions to form **4.26** and **4.31** were low-yielding involving longer reaction times because of the presence of the electron withdrawing nitro group in the ring (Schemes 4.5 and 4.6). Boc deprotection of the N-oxides by TFA followed by hydrogenation over Pd/C afforded **4.11** and **4.12**.

Compounds **4.1-4.12** are inhibitors of mitochondrial electron transfer at all tested concentrations. Analogues with longer alkyl side chains were generally found to be less inhibitory than the corresponding analogues having shorter side chains (Table 4.1). However, 16 carbon chain compound **4.12** having a polar amine group on the redox core was a strong inhibitor even at lower concentrations (Table 4.2).

As shown in Table 4.3, all of the new *N*-hydroxy-4-pyridones were tested for their ability to quench lipid peroxidation in FRDA lymphocytes following oxidative challenge with diethyl maleate. Most of the analogues were inactive or poorly effective in suppressing lipid peroxidation. Increasing the side chain length (to 16 carbon atoms)

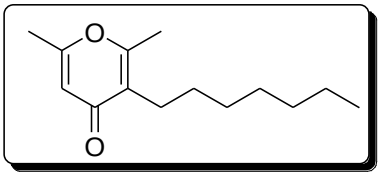
improved suppression of lipid peroxidation. The analogues were tested at two different (2.5 and 5 μM) concentrations and compound **4.12** having electron-donating amine group on the redox core was the best inhibitor of lipid peroxidation (94% suppression at 5 μM concentration). However, at a lower concentration, it did not show much suppression. Some of the compounds were also tested for suppression of ROS in CEM lymphocytes (Table 4.4), affording results that fairly closely paralleled those found for lipid peroxidation. The analogues were tested at three different (0.25, 2.5 and 10.0 μM) concentrations; the results indicated that amino-substituted analogues **4.11** and **4.12** suppressed ROS reasonably potently at high (10 μM) concentration. The ability of the synthesized *N*-hydroxy-4-pyridones to protect cultured Friedreich's ataxia lymphocytes from cell death by oxidative stress was measured (Table 4.5). None of the compounds provided significant cytoprotection from DEM-induced stress in FRDA lymphocytes.

The biological results provide an understanding of the structure-activity relationships between the *N*-hydroxy-4-pyridone analogues and their binding to the mitochondrial complexes. Analogue **4.12** exhibited promising biological activity in inhibition of lipid peroxidation and ROS suppression. However, its inhibitory effect towards the respiratory chain as well as its inability to confer cytoprotection to cultured FRDA cells makes it a poor candidate for treating mitochondrial disorders. Therefore, further studies and modification of these *N*-hydroxy-4-pyridones will be required to identify a potential candidate for the treatment of Friedreich's ataxia and other disorders of the mitochondrial respiratory chain.

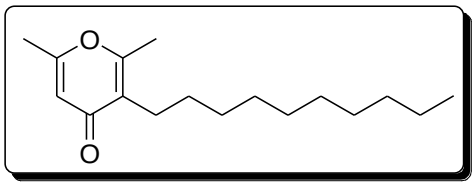
4.4. Experimental Procedures

The chemicals used were purchased from Alfa Aesar, Aldrich Chemical Co. or Sigma Chemical Co. and were used without further purification. Tetrahydrofuran and dichloromethane were distilled from sodium/benzophenone and calcium hydride, respectively. All experiments requiring anhydrous conditions were conducted in flame-dried glassware fitted with rubber septa under a positive pressure of dry nitrogen or argon. Reactions were performed at room temperature unless indicated otherwise. Analytical thin layer chromatography was performed using glass plates pre-coated with silica gel (0.25 mm, 60 Å pore size, 230-400 mesh, Silicycle) impregnated with a fluorescent indicator (254 nm). TLC chromatograms were visualized by exposure to ultraviolet light (UV). Flash column chromatography was performed employing silica gel (60 Å pore size, 40-63 μm, standard grade, Silicycle).

^{1}H NMR and ^{13}C NMR spectra were recorded on Varian INOVA 400 (400 MHz) or Varian INOVA 500 (500 MHz) spectrometers at 25 °C. Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to residual protium in the NMR solvent ($CDCl_3$ or CD_3OD). Splitting patterns are designated as follows: s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or the Michigan State University Mass Spectrometry Facility.

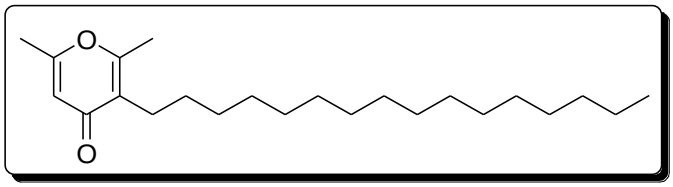


3-Heptyl-2,6-dimethyl-4*H*-pyran-4-one (4.13). To a mixture of 49.0 g of polyphosphoric acid and 30 mL of acetic acid was added 0.60 mL (0.50 g, 3.20 mmol) of 2-decanone. The resulting solution was heated at 130 °C with vigorous stirring for 8 h. The resulting dark black solution was cooled to 0 °C, diluted with 200 mL of water and extracted with two 100-mL portions of EtOAc. The combined organic phase was washed with 10% aq KOH, dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (20 × 4 cm). Elution with 1:1 hexane–ethyl acetate gave 3-heptyl-2,6-dimethyl-4*H*-pyran-4-one (**4.13**) as a light yellow solid: yield 555 mg (51%); silica gel TLC R_f 0.45 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.45-0.50 (m, 3H), 0.88-1.04 (m, 10H), 1.82 (s, 3H), 1.89 (s, 3H), 1.98 (t, 2H, J = 7.6 Hz) and 5.62 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 16.6, 18.9, 21.9, 23.6, 27.8, 28.5, 28.9, 31.1, 112.1, 124.0, 160.6, 163.5 and 178.4; mass spectrum (APCI), *m/z* 223.1696 $(M + H)^+$ ($C_{14}H_{23}O_2$ requires *m/z* 223.1698).



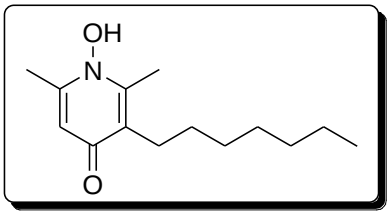
3-Decyl-2,6-dimethyl-4*H*-pyran-4-one (4.14). To a mixture of 50.0 g of polyphosphoric acid and 30 mL of acetic acid was added 0.61 mL (0.50 g, 2.52 mmol) of 2-tridecanone. The resulting solution was heated at 130 °C with vigorous stirring for 8 h. The resulting dark black solution was cooled to 0 °C, diluted with 200 mL of water and extracted with two 100-mL portions of EtOAc. The combined organic phase was washed with 10% aq KOH, dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford

a crude residue. The residue was purified by chromatography on a silica gel column (20 × 4 cm). Elution with 1:1 hexane–ethyl acetate gave 3-decyl-2,6-dimethyl-4*H*-pyran-4-one (**4.14**) as a light yellow solid: yield 331 mg (49%); silica gel TLC R_f 0.52 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.80-0.83 (m, 3H), 1.12-1.37 (m, 16H), 2.14 (s, 3H), 2.21 (s, 3H), 2.30-2.34 (t, 2H, J = 8.0 Hz) and 6.00 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 17.4, 19.7, 22.7, 24.5, 28.6, 29.4, 29.6, 29.7, 29.8, 32.0, 113.0, 124.9, 161.5, 164.3 and 179.6; mass spectrum (APCI), *m/z* 265.2163 $(M + H)^+$ ($C_{17}H_{29}O_2$ requires *m/z* 265.2168).

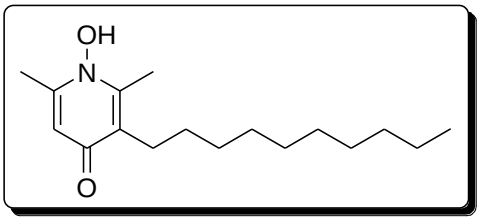


3-Hexadecyl-2,6-dimethyl-4*H*-pyran-4-one (4.15). To a mixture of 49.5 g of polyphosphoric acid and 30 mL of acetic acid was added 0.50 g (1.77 mmol) of 2-nonadecanone. The resulting solution was heated at 130 ^{o}C with vigorous stirring for 8 h. The resulting dark black solution was cooled to 0 ^{o}C, diluted with 200 mL of water and extracted with two 100-mL portions of EtOAc. The combined organic phase was washed with 10% aq KOH, dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (20 × 4 cm). Elution with 2:1 hexane–ethyl acetate gave 3-hexadecyl-2, 6-dimethyl-4*H*-pyran-4-one (**4.15**) as a light yellow solid: yield 331 mg (45%); silica gel TLC R_f 0.60 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.78-0.82 (m, 3H), 1.18-1.24 (m, 28H), 2.14 (s, 3H), 2.21 (s, 3H), 2.30 (t, 2H, J = 8.4 Hz) and 6.06 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 17.5, 19.8, 22.8, 24.5, 28.7, 29.5, 29.7, 29.78,

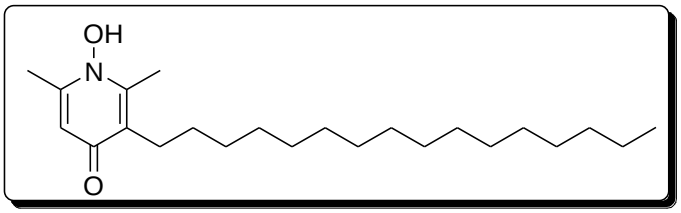
29.81, 29.83, 32.1, 113.1, 125.1, 161.6, 164.4 and 179.7; mass spectrum (APCI), *m/z* 349.3106 $(M + H)^+$ ($C_{23}H_{41}O_2$ requires *m/z* 349.3107).



3-Heptyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.1). A mixture containing 113 mg (0.51 mmol) of trimethyl pyrone **4.13**, 707 mg (10.2 mmol) of hydroxylamine hydrochloride, 835 mg (10.2 mmol) of sodium acetate, 1.0 mL of water and 2.0 mL of EtOH was heated at reflux for 18 h. The cooled reaction mixture filtered, and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 5:1 ethyl acetate–methanol gave 3-heptyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (**4.1**) as a brown solid: yield 62.9 mg (50%); silica gel TLC R_f 0.35 (3:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.84-0.87 (m, 3H), 1.26-1.45 (m, 10H), 2.42 (s, 3H), 2.48 (s, 3H), 2.59-2.63 (t, 2H, J = 8.4 Hz), 5.21 (br s, 1H) and 6.73 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.6, 14.8, 18.5, 23.7, 27.1, 29.9, 30.3, 30.6, 33.0, 111.6, 126.4, 148.9, 150.3 and 160.1; mass spectrum (APCI), *m/z* 238.1808 $(M + H)^+$ ($C_{14}H_{24}NO_2$ requires *m/z* 238.1807).

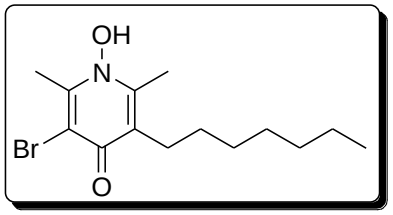


3-Decyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.2). A mixture containing 200 mg (0.75 mmol) of trimethyl pyrone **4.14**, 1.05 g (15.2 mmol) of hydroxylamine hydrochloride, 1.24 g (15.2 mmol) of sodium acetate, 1.0 mL of water and 2.0 mL of EtOH was heated at reflux for 3 days. The cooled reaction mixture was concentrated under dimished pressure to afford a brown residue. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 5:1 ethyl acetate–methanol gave 3-decyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (**4.2**) as a brown oil: yield 90.1 mg (49%); silica gel TLC R_f 0.41 (3:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.85-0.88 (m, 3H), 1.26-1.46 (m, 16H), 2.42 (s, 3H), 2.49 (s, 3H), 2.61-2.65 (t, 2H, J = 8.0 Hz) and 6.74 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.5, 14.8, 18.5, 23.8, 27.1, 29.9, 30.0, 30.5, 30.56, 30.61, 30.8, 33.1, 111.5, 126.4, 149.1, 150.5 and 160.0; mass spectrum (APCI), *m/z* 280.2282 $(M + H)^+$ ($C_{17}H_{30}NO_2$ requires *m/z* 280.2277).

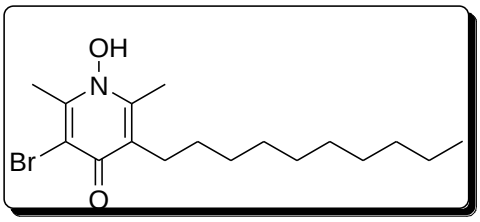


3-Hexadecyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.3). A mixture containing 200 mg (0.57 mmol) of trimethyl pyrone **4.15**, 399 mg (5.70 mmol) of hydroxylamine hydrochloride, 471 mg (5.70 mmol) of sodium acetate, 1.0 mL of water and 2.0 mL of EtOH was heated at reflux for 3 days. The cooled reaction mixture was concentrated under diminished pressure to afford a brown oil. The residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 5:1 ethyl acetate–methanol gave 3-hexadecyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (**4.3**) as a brown

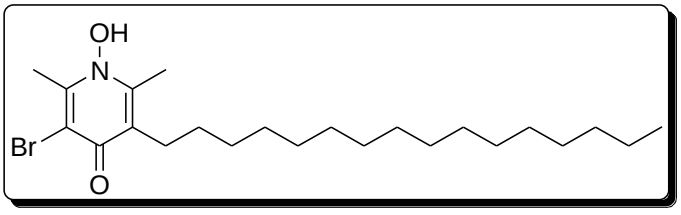
solid: yield 89.2 mg (43%); silica gel TLC R_f 0.45 (3:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.83-0.85 (m, 3H), 1.22-1.27 (m, 28H), 2.32 (s, 3H), 2.46 (s, 3H), 2.55 (t, 2H, J = 8.4 Hz) and 6.70 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.1, 16.8, 18.8, 22.5, 22.6, 25.3, 28.9, 29.3, 29.6, 29.7, 30.0, 31.9, 113.4, 126.1, 145.1, 146.8 and 178.4; mass spectrum (APCI), *m/z* 364.3212 $(M + H)^+$ ($C_{23}H_{41}O_2$ requires *m/z* 364.3216).



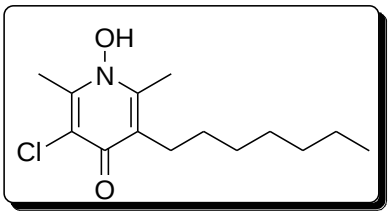
3-Bromo-5-heptyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.4). To a stirred solution containing 63.0 mg (0.27 mmol) of **4.1** in 2.0 mL acetic acid was added dropwise a solution containing 0.04 mL (0.27 mmol) of bromine in 1.0 mL of acetic acid at room temperature. After 2 h a few drops of a saturated aq sodium sulfite was added to discharge excess bromine. The reaction mixture was diluted with 20 mL of water and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave compound 3-bromo-5-heptyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (**4.4**) as a yellow oil: yield 57.4 mg (69%); silica gel TLC R_f 0.68 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.84-0.87 (m, 3H), 1.26-1.41 (m, 10H), 2.41 (s, 3H), 2.57-2.61 (m, 2H), 2.63 (s, 3H) and 4.86 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.4, 14.7, 18.8, 23.2, 27.7, 29.3, 29.8, 30.3, 32.4, 111.5, 126.2, 147.0, 147.6 and 166.7; mass spectrum (APCI), *m/z* 316.0911 $(M + H)^+$ ($C_{14}H_{23}NO_2Br$ requires *m/z* 316.0912).



3-Bromo-5-decyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.5). To a stirred solution containing 40.0 mg (0.14 mmol) of **4.2** in acetic acid (2.0 mL) was added dropwise a solution containing 0.02 mL (0.14 mmol) of bromine in 1.0 mL of acetic acid at room temperature. After 2 h a few drops of a saturated aq sodium sulfite was added to discharge excess bromine. The reaction mixture was diluted with 20 mL of water and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave compound 3-bromo-5-decyl-1-hydroxy-2, 6-dimethylpyridin-4(1*H*)-one (**4.5**) as a brown oil: yield 37.0 mg (73%); silica gel TLC R_f 0.75 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88-0.91 (m, 3H), 1.29-1.46 (m, 16H), 2.44 (s, 3H) and 2.66 (s, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 14.9, 19.0, 22.8, 28.9, 29.5, 29.7, 29.79, 29.82, 32.1, 111.7, 126.0, 146.8, 147.6 and 168.9; mass spectrum (APCI), *m/z* 358.1381 $(M + H)^+$ ($C_{17}H_{29}NO_2Br$ requires *m/z* 358.1382).

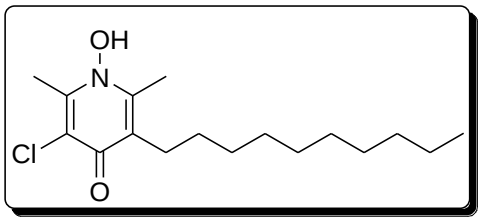


3-Bromo-5-hexadecyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.6). To a stirred solution containing 44.0 mg (0.12 mmol) of **4.3** in 2.0 mL acetic acid was added dropwise a solution containing 0.01 mL (0.12 mmol) of bromine in 1.0 mL of acetic acid at 10-15 ^{o}C. After 1.5 h a few drops of a saturated aq sodium sulfite was added to discharge excess bromine. The reaction mixture was diluted with 20 mL of water and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave compound 3-bromo-5-hexadecyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (**4.6**) as a brown solid: yield 38.9 mg (71%); silica gel TLC R_f 0.80 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400MHz) δ 0.87-0.89 (m, 3H), 1.25-1.51 (m, 28H) and 2.41-2.58 (m, 8H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.2, 15.9, 18.8, 22.3, 22.6, 25.3, 27.9, 29.3, 29.2, 29.7, 30.0, 30.9, 113.4, 127.1, 145.2, 147.8 and 177.1; mass spectrum (APCI), *m/z* 442.2325 $(M + H)^+$ ($C_{23}H_{41}NO_2Br$ requires *m/z* 442.2321).



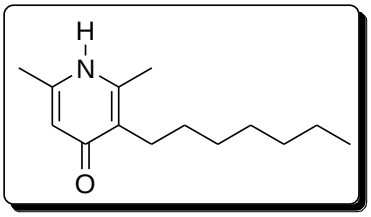
3-Chloro-5-heptyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.7). To a stirred solution containing 11.0 mg (0.05 mmol) of **4.1** in 1.0 mL of acetic acid was added 6.70 mg (0.05 mmol) of *N*-chlorosuccinimide. The reaction mixture was heated at 100 °C for 8 h and cooled to room temperature, diluted with 20 mL of water and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous

$MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave compound 3-chloro-5-heptyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (**4.7**) as a brown solid: yield 7.10 mg (54%); silica gel TLC R_f 0.66 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88-0.91 (m, 3H), 1.26-1.45 (m, 10H), 2.34 (s, 3H), 2.43 (s, 3H) and 2.48-2.55 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.4, 14.7, 18.8, 23.2, 27.7, 29.3, 29.8, 30.3, 32.4, 111.5, 126.2, 147.0, 147.6 and 166.7; mass spectrum (APCI), *m/z* 272.1422 $(M + H)^+$ ($C_{14}H_{23}NO_2Cl$ requires *m/z* 272.1417).

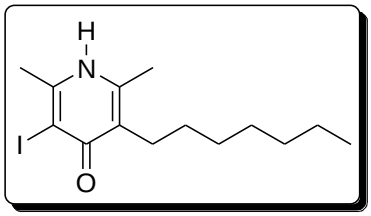


3-Chloro-5-decyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.8). To a stirred solution containing 40.0 mg (0.14 mmol) of **4.2** in 2.0 mL of acetic acid was added 19.1 mg (0.14 mmol) of *N*-chlorosuccinimide. The reaction mixture was heated at 100 °C for 8 h and cooled to room temperature, diluted with 20 mL of water and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave 3-chloro-5-decyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (**4.8**) as a brown solid: yield 28.3 mg (64%); silica gel TLC R_f 0.7 (7:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.73-0.78 (m, 3H), 1.15-1.20 (m,

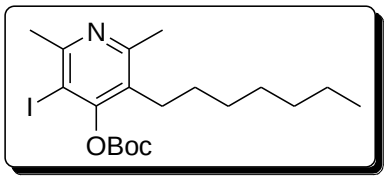
16H), 2.25 (s, 3H), 2.30 (s, 3H) and 2.37-2.40 (m, 2H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.5, 14.8, 18.5, 23.8, 27.1, 29.9, 30.0, 30.5, 30.56, 30.61, 30.8, 33.1, 111.5, 126.4, 149.1, 150.5 and 160.0; mass spectrum (APCI), *m/z* 314.1877 $(M + H)^+$ ($C_{17}H_{29}NO_2Cl$ requires *m/z* 314.1887).



3-Heptyl-2,6-dimethylpyridin-4(1*H*)-one (4.16). Trimethyl-γ-pyrone **4.13** (0.01 g, 30.6 mmol) was heated at 100 °C with 2 mL of aqueous ammonia in a high pressure tube for 18 h. The cooled reaction mixture was concentrated under diminished pressure to afford a brown oil. The crude residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave 3-heptyl-2, 6-dimethylpyridin-4(1*H*)-one (**4.16**) as a brown solid: yield 50.7 mg (51%); silica gel TLC R_f 0.25 (3:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.88-0.90 (m, 3H), 1.29-1.46 (m, 10H), 2.30 (s, 3H), 2.36 (s, 3H), 2.48-2.52 (m, 2H), 4.97 (br s, 1H) and 6.25 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.5, 17.1, 18.8, 23.8, 26.0, 29.8, 30.5, 30.9, 33.1, 114.4, 127.2, 147.0, 148.6 and 179.6; mass spectrum (APCI), *m/z* 222.1850 $(M + H)^+$ ($C_{14}H_{24}NO$ requires *m/z* 222.1858).

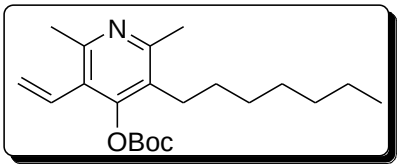


3-Heptyl-5-iodo-2,6-dimethylpyridin-4(1*H*)-one (4.17). To a stirred solution containing 100 mg (0.45 mmol) of compound **4.16** in acetonitrile (2.5 mL) was added 25.0 mg (45.0 μmol) of ceric ammonium nitrate followed by 126 mg (50 μmol) of iodine. The reaction mixture was heated at 70 ^{o}C under nitrogen for 7 h. The resulting dark brown solution was cooled to room temperature, diluted with 200 mL of water and extracted with two 100-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexane–ethyl acetate gave 3-heptyl-5-iodo-2,6-dimethylpyridin-4(1*H*)-one (**4.17**) as a light yellow solid: yield 110 mg (68%); silica gel TLC R_f 0.45 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88-0.91 (m, 3H), 1.29-1.46 (m, 10H), 2.34 (s, 3H) and 2.53-2.56 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 16.6, 18.9, 21.9, 23.6, 27.8, 28.5, 28.9, 31.1, 112.1, 124.0, 160.6, 163.5 and 178.4; mass spectrum (APCI), *m/z* 348.0835 $(M + H)^+$ ($C_{14}H_{23}INO$ requires *m/z* 348.0825).



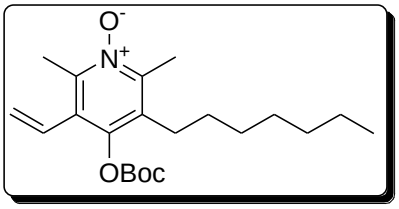
***tert*-Butyl-3-heptyl-5-iodo-2,6-dimethylpyridin-4-yl carbonate (4.18).** To a stirred solution containing 60.0 mg (0.73 mmol) of iodo derivative **4.17** in anhydrous THF (3 mL) was added 102 mg (0.91 mmol) of *t*-BuOK and the reaction mixture was stirred at room temperature for 1 h. Then 175 mg (0.80 mmol) of Boc anhydride was added and the reaction mixtre was heated at 60 °C for 1 h. The cooled reaction mixture was concentrated under diminished pressure to afford a yellow oil. The residue was purified

by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 hexane–ethyl acetate gave **4.18** as a light yellow oil: yield 60.1 mg (75%); silica gel TLC R_f 0.71 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.82-0.86 (m, 3H), 1.22-1.33 (m, 10H), 1.51 (s, 9H) and 2.51-2.54 (m, 8H); mass spectrum (APCI), *m/z* 448.1355 $(M + H)^+$ ($C_{19}H_{31}INO_3$ requires *m/z* 448.1349).

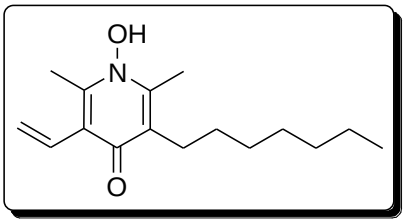


***tert*-Butyl 3-heptyl-2,6-dimethyl-5-vinylpyridin-4-yl carbonate (4.19).** To a stirred solution containing 60.0 mg (0.864 mmol) of **4.18** in 1,4-dioxane (4 mL) were added 73.2 mg (1.73 mmol) of lithium chloride, 99.8 mg (10 mol%) of tetrakis(triphenylphosphine)palladium and 0.33 mL (355 mg, 1.12 mmol) of tributyl(vinyl)tin. The reaction mixture was heated at reflux for 8 h and cooled to room temperature, diluted with 20 mL of water and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave compound **4.19** as a light yellow oil: yield 12.5 mg (25%); silica gel TLC R_f 0.25 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.81-0.85 (m, 3H), 1.24-1.42 (m, 10H), 1.47 (s, 9H), 2.44 (s, 3H), 2.45-2.47 (m, 5H), 5.48 (dd, 2H, J = 18 and 11.2 Hz) and 6.51 (dd, 1H, J = 17.8 and 11.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 22.2, 22.7, 23.0, 26.6, 27.7, 29.08, 29.10, 29.9, 31.8, 83.7, 120.7, 123.9, 126.4,

129.7, 150.5, 153.8, 154.4 and 156.5; mass spectrum (APCI), *m/z* 348.2536 $(M + H)^+$ ($C_{21}H_{34}NO_3$ requires *m/z* 348.2539).

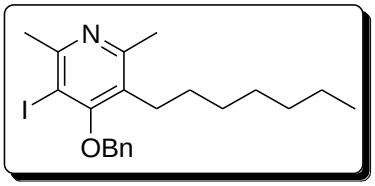


***tert*-Butyl 3-heptyl-2,6-dimethyl-5-vinylpyridin-4-yl carbonate-N-oxide (4.20).** To a stirred solution containing 30.0 mg (0.66 mmol) of compound **4.19** in 2 mL of anhydrous CH_2Cl_2, 125 mg (0.72 mmol) of mCPBA was added. The reaction mixture was stirred at 0 °C for 1 h under argon atmosphere, and then concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave *tert*-butyl-3-heptyl-2,6-dimethyl-5-vinylpyridin-4-yl-carbonate-*N*-oxide (**4.20**) as a light yellow oil: yield 23.4 mg (76%); silica gel TLC R_f 0.25 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.80-0.84 (m, 3H), 1.22-1.28 (m, 10H), 1.45 (s, 9H), 2.46 (s, 3H), 2.47-2.49 (m, 5H), 5.48 (d, 1H, J = 17.6 Hz), 5.56 (d, 1H, J = 11.6 Hz) and 6.51 (dd, 1H, J = 17.8 and 11.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 14.9, 15.6, 22.7, 27.2, 27.6, 29.0, 29.2, 29.6, 31.8, 84.3, 122.9, 127.0, 128.4, 129.3, 143.7, 146.3, 148.1 and 150.5; mass spectrum (APCI), *m/z* 364.2480 $(M + H)^+$ ($C_{21}H_{34}NO_4$ requires *m/z* 364.2488).



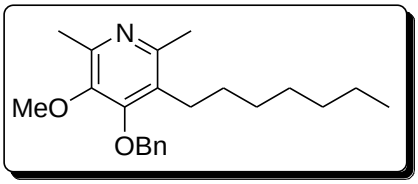
3-Heptyl-1-hydroxy-2, 6-dimethyl-5-vinylpyridin-4(1*H*)-one (4.9). To a stirred solution containing 138 mg (0.54 mmol) of compound **4.20** in 1.5 mL of EtOH, aqueous

10 M KOH was added. The reaction mixture was stirred at room temperature for 1 h, 3 mL of water was added and pH was brought to 1-2 using conc. HCl, extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave 3-heptyl-1-hydroxy-2,6-dimethyl-5-vinylpyridin-4(1*H*)-one (**4.9**) as a light yellow oil: yield 12.7 mg (72%); silica gel TLC R_f 0.35 (1:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87-0.91 (m, 3H), 1.29-1.50 (m, 10H), 2.49 (s, 3H), 2.50 (s, 3H), 2.65-2.69 (m, 2H), 5.57 (d, 1H, J = 17.6 Hz), 5.68 (d, 1H, J = 11.6 Hz) and 6.64 (dd, 1H, J = 17.8 and 11.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.5, 14.8, 15.8, 23.7, 27.3, 30.1, 30.3, 30.7, 33.0, 123.4, 123.6, 126.6, 130.4, 147.1 and 148.4; mass spectrum (APCI), *m/z* 264.1956 $(M + H)^+$ ($C_{16}H_{26}NO_2$ requires *m/z* 264.1964).



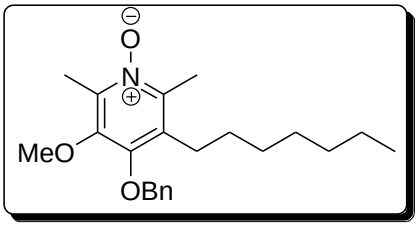
4-(Benzyloxy)-3-heptyl-5-iodo-2,6-dimethylpyridine (4.21). To a stirred suspension of oil-free NaH (5.0 mg, 0.11 mmol) in 5 mL of DMF was added 38.0 mg (0.11 mmol) of compound **4.17**. When evolution of hydrogen ceased, 14.0 mg (0.11 mmol) of benzyl chloride was added and the reaction mixture was heated at 50 °C for 4 h. The cooled reaction mixture was diluted with 10 mL of water and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was

purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:1 hexane–ethyl acetate gave 4-(benzyloxy)-3-heptyl-5-iodo-2,6-dimethylpyridine (**4.21**) as a light yellow oil: yield 38.0 mg (80%); silica gel TLC R_f 0.7 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.85-0.88 (m, 3H), 1.26-1.33 (m, 8H), 1.48-1.51 (m, 2H), 2.50 (s, 3H), 2.60-2.64 (m, 2H) 2.74 (s, 3H), 4.95 (s, 2H), 7.37-7.43 (m, 4H) and 7.55-7.57 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 21.9, 22.7, 27.4, 29.1, 29.4, 29.8, 30.0, 31.9, 75.1, 91.7, 128.0, 128.7, 136.5, 158.0, 159.0 and 163.7; mass spectrum (APCI), *m/z* 438.1303$(M + H)^+$ ($C_{21}H_{29}NOI$ requires *m/z* 438.1294).

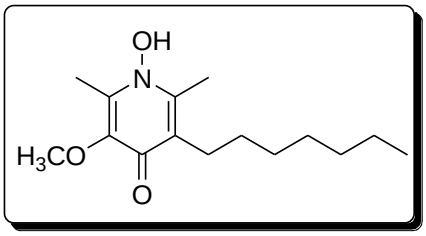


4-(Benzyloxy)-3-heptyl-5-methoxy-2,6-dimethylpyridine (4.22). To a stirred solution containing sodium methoxide prepared from 5.10 mg (0.23 mmol) of sodium and 1 mL of MeOH were added 27.0 mg (0.06 mmol) of the iodo derivative **4.21** and 1.66 mg (0.01 mmol) of CuI. The reaction mixture was heated at 110 °C for 18 h. The cooled reaction mixture was diluted with 1 M aqueous NH_4Cl and extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave 4-(benzyloxy)-3-heptyl-5-methoxy-2,6-dimethylpyridine (**4.22**) as a light yellow oil: yield 19.0 mg (71%); silica gel TLC R_f 0.3 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.85-0.87 (m, 3H), 1.23-1.38 (m, 10H), 2.41 (s, 3H), 2.43 (s, 3H), 2.48-2.52 (m,

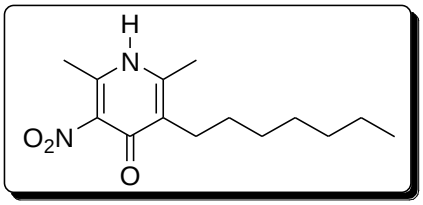
2H), 3.76 (s, 3H), 5.11 (s, 2H) and 7.33-7.43 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 18.7, 22.0, 22.7, 26.6, 29.1, 29.7, 30.0, 31.9, 60.3, 64.8, 74.5, 127.0, 128.0, 128.5, 137.4, 145.4, 150.0, 152.4 and 148.4; mass spectrum (APCI), *m/z* 342.2431 $(M + H)^+$ ($C_{22}H_{32}NO_2$ requires *m/z* 342.2433).



4-(Benzyloxy)-3-heptyl-5-methoxy-2,6-dimethylpyridine-N-oxide (4.23). To a stirred solution containing 19.0 mg (0.06 mmol) of compound **4.22** in 2 mL of anhydrous CH_2Cl_2 was added 12.5 mg (0.07 mmol) of mCPBA. The reaction mixture was stirred at 0 °C for 1 h under argon atmosphere, and then concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave 4-(benzyloxy)-3-heptyl-5-methoxy-2,6-dimethylpyridine-*N*-oxide (**4.23**) as a light yellow oil: yield 17.3 mg (87%); silica gel TLC R_f 0.25 (10:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.84-0.86 (m, 3H), 1.23-1.36 (m, 10H), 2.47 (s, 3H), 2.50 (s, 3H), 2.53-2.56 (m, 2H), 3.81 (s, 3H), 5.08 (s, 2H) and 7.34-7.40 (m, 5H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 11.9, 14.2, 14.7, 22.7, 26.8, 29.1, 31.9, 61.1, 75.3, 128.3, 128.7, 129.9, 136.8, 143.3, 145.3, 146.7 and 148.3; mass spectrum (APCI), *m/z* 358.2385 $(M + H)^+$ ($C_{22}H_{32}NO_3$ requires *m/z* 358.2382).

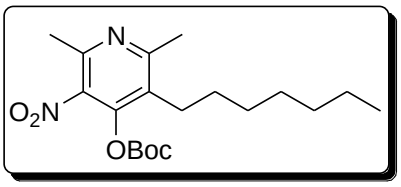


3-Heptyl-1-hydroxy-5-methoxy-2,6-dimethylpyridin-4(1*H*)-one (4.10). To a solution containing 18.0 mg (0.05 mmol) of **4.23** in 5 mL of EtOH was added catalytic amount of 10% Pd/C and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and the filtrate was concentrated under diminished pressure and purified by chromatography on a silica gel column (10 × 1 cm). Elution with 5:1 ethyl acetate–methanol gave **4.10** as a colorless solid: yield 9.91 mg (70%); silica gel TLC R_f 0.21 (5:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89-0.91 (m, 3H), 1.31-1.51 (m, 10H), 2.46 (s, 3H), 2.48 (s, 3H), 2.69-2.72 (m, 2H) and 3.75 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 12.0, 14.4, 14.6, 23.7, 27.0, 30.0, 30.3, 30.6, 33.0, 61.9, 111.4, 126.0, 143.5, 143.9 and 146.7; mass spectrum (EI), *m/z* 267.1838 $(M)^+$ ($C_{15}H_{25}NO_3$ requires *m/z* 267.1834).

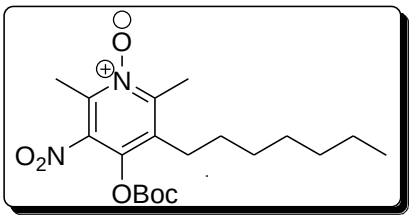


3-Heptyl-2,6-dimethyl-5-nitropyridin-4(1*H*)-one (4.24). To a stirred solution containing 94.0 mg (0.41) of compound **4.16** in 1 mL of sulfuric acid (d 1.64, 0.5 g) at 5-10 °C was added a mixture of 0.10 mL (155 mg, 2.46 mmol) of nitric acid and 0.05 mL (80.3 mg, 0.82 mmol) of sulfuric acid. The reaction mixture was heated at 80 °C for 3 h. The cooled reaction mixture was added to 100 mL of ice water. Saturated aqueous sodium carbonate was added until effervescence ceased. The reaction mixture was extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm).

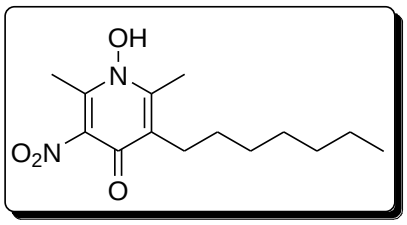
Elution with ethyl acetate gave 3-heptyl-2,6-dimethyl-5-nitropyridin-4(1*H*)-one (**4.24**) as a yellow oil: yield 76.1 mg (68%); silica gel TLC R_f 0.45 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.84-0.88 (m, 3H), 1.25-1.32 (m, 8H), 1.42-1.44 (m, 2H), 2.35 (s, 3H), 2.42 (s, 3H) and 2.49-2.52 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 16.1, 17.1, 22.8, 25.8, 28.6, 29.3, 30.0, 31.9, 129.9, 140.2, 143.3, 145.8 and 160.1; mass spectrum (APCI), *m/z* 267.1708 $(M+H)^+$ ($C_{15}H_{23}NO_3$ requires *m/z* 267.1709).



***tert*-Butyl-3-heptyl-2,6-dimethyl-5-nitropyridin-4-yl carbonate (4.25).** To a stirred solution containing 76.0 mg (0.29 mmol) of nitro derivative **4.24** in 3 mL of anhydrous THF was added 40.1 mg (0.36 mmol) of *t*-BuOK. The reaction mixture was stirred at room temperature for 1 h. Then 63.0 mg (0.32 mmol) of Boc_2O was added and the reaction mixture was heated at 60 °C for 5 h. The cooled reaction mixture was concentrated under diminished pressure to afford a yellow oil. The crude residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 hexane–ethyl acetate gave *tert*-butyl 3-heptyl-2,6-dimethyl-5-nitropyridin-4-yl carbonate (**4.25**) as a light yellow oil: yield 54.1 mg (53%); silica gel TLC R_f 0.7 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.85-0.88 (m, 3H), 1.24-1.32 (m, 10H), 1.54 (s, 9H) and 2.54-2.57 (m, 8H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 21.5, 22.7, 23.0, 26.7, 27.6, 28.7, 29.0, 29.8, 31.8, 85.8, 128.9, 139.3, 148.1, 149.5, 149.6 and 161.4; mass spectrum (APCI), *m/z* 367.2230 $(M+H)^+$ ($C_{19}H_{31}N_2O_5$ requires *m/z* 367.2233).

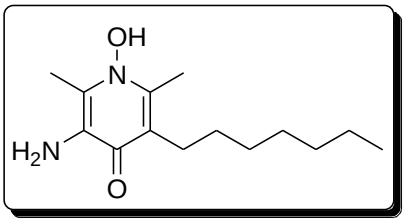


***tert*-Butyl 3-heptyl-2,6-dimethyl-5-nitropyridin-*N*-oxide-4-yl carbonate (4.26).** To a stirred solution containing 28.0 mg (0.08 mmol) of compound **4.25** in 2 mL of anhydrous CH_2Cl_2 was added 15.0 mg (0.09 mmol) of mCPBA. The reaction mixture was stirred at 0 °C for 1 h under argon atmosphere, then concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave *tert*-butyl 3-heptyl-2,6-dimethyl-5-nitropyridin-*N*-oxide -4-yl-carbonate (**4.26**) as a light brown solid: yield 21.3 mg (72%); silica gel TLC R_f 0.40 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.82-0.85 (m, 3H), 1.24-1.34 (m, 8H), 1.45-1.46 (m, 2H), 1.47 (s, 9H), 2.50 (s, 3H), 2.54 (s, 3H) and 2.56-2.58 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 14.5, 15.5, 22.7, 27.4, 27.5, 28.8, 28.9, 29.6, 31.7, 128.9, 131.4, 137.0, 141.1, 142.6, 149.7 and 152.8; mass spectrum (APCI), *m/z* 383.2178 $(M+H)^+$ ($C_{19}H_{31}N_2O_6$ requires *m/z* 383.2182).



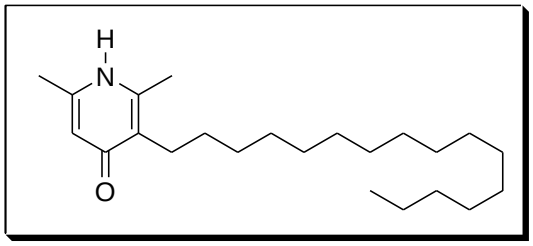
3-Heptyl-1-hydroxy-2,6-dimethyl-5-nitropyridin-4(1*H*)-one (4.27). To a stirred solution containing 115 mg (0.30 mmol) of **3.16** in 2 mL of anhydrous CH_2Cl_2 was added 0.23 mL (342 mg, 3.00 mmol) of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 4 h, then diluted with 50 mL of satd aq $NaHCO_3$ and extracted with

two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave 3-Heptyl-1-hydroxy-2,6-dimethyl-5-nitropyridin-4(1*H*)-one (**4.27**) as a light brown solid: yield 66.1 mg (78%); silica gel TLC R_f 0.30 (10:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.85-0.89 (m, 3H), 1.27-1.33 (m, 8H), 1.43-1.45 (m, 2H), 2.54 (s, 3H), 2.55 (s, 3H) and 2.61-2.64 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 14.5, 15.1, 22.8, 26.8, 28.6, 29.2, 29.8, 31.9, 129.8, 138.0, 145.3, 151.2 and 162.8; mass spectrum (APCI), *m/z* 283.1661 $(M+H)^+$ ($C_{14}H_{23}N_2O_4$ requires *m/z* 283.1658).

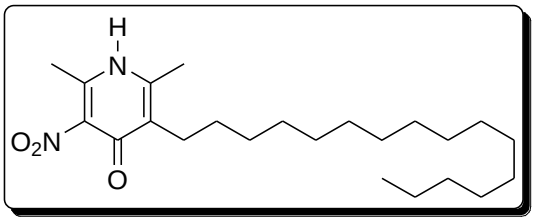


3-Amino-5-heptyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.11). To a solution containing 64.0 mg (0.23 mmol) of **4.27** in 2.5 mL of EtOH was added catalytic amount of 10% Pd/C and the reaction was placed under 1 atm of H_2 (g) for 30 min. The catalyst was removed by filtration through a pad of Celite 545® and the filtrate was concentrated under diminished pressure and purified by chromatography on a silica gel column(10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave 3-amino-5-heptyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (**4.11**) as a light yellow oil: yield 45.1 mg (79%); silica gel TLC R_f 0.25 (3:1 ethyl acetate–methanol); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.86-0.88 (m, 3H), 1.27-1.43 (m, 10H), 2.41 (s, 6H) and 2.61-2.63 (m, 2H); ^{13}C NMR (CD_3OD, 100 MHz) δ 12.3, 14.2, 14.4, 23.4, 27.1, 30.0, 30.1, 30.4, 32.7, 111.0, 123.7, 132.6, 133.5 and

162.8; mass spectrum (APCI), *m/z* 253.1908 $(M+H)^+$ ($C_{14}H_{25}N_2O_2$ requires *m/z* 253.1916).

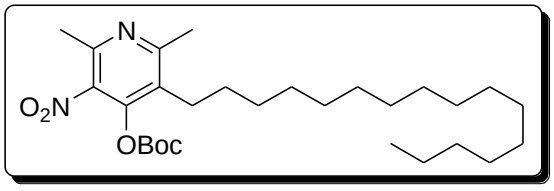


3-Hexadecyl-2,6-dimethylpyridin-4(1*H*)-one (4.28). Trimethyl-γ-pyrone **4.15** (0.02 g, 30.6 mmol) was heated at 100 °C with 2 mL of aqueous ammonia in a high pressure tube for 18 h. The cooled reaction mixture was concentrated under diminished pressure. The crude residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:1 ethyl acetate–methanol gave 3-hexadecyl-2, 6-dimethylpyridin-4(1*H*)-one (**4.28**) as a brown solid: yield 39.8 mg (40%); silica gel TLC R_f 0.25 (3:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.81-0.84 (m, 3H), 1.21-1.40 (m, 28H), 2.24 (s, 3H), 2.34 (s, 3H), 2.45-2.52 (m, 2H) and 6.07 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.2, 16.8, 18.8, 22.7, 25.4, 28.9, 29.4, 29.7, 29.8, 30.1, 32.0, 113.5, 126.2, 145.2, 146.8 and 178.5; mass spectrum (APCI), *m/z* 348.3271 $(M+H)^+$ ($C_{14}H_{25}N_2O_2$ requires *m/z* 348.3266).



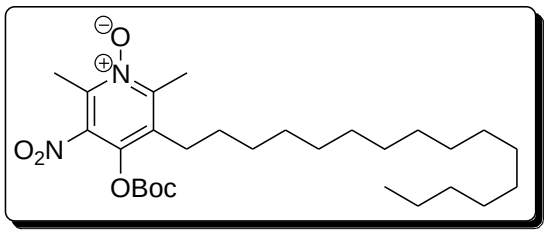
3-Hexadecyl-2,6-dimethyl-5-nitropyridin-4(1*H*)-one (4.29). To a stirred solution containing 69.6 mg (0.20) of compound **4.28** in 1 mL of sulfuric acid at 5-10 °C, a mixture of 0.10 mL of nitric acid (155 mg, 2.46 mmol) and 0.05 mL (80.3 mg, 0.82

mmol) of sulfuric acid was added. The reaction mixture was heated at 80°C for 3 h. The cooled reaction mixture was added to ice. Saturated aqueous sodium carbonate was added until effervescence ceased. The reaction mixture was extracted with two 20-mL portions of EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave 3-hexadecyl-2,6-dimethyl-5-nitropyridin-4(1*H*)-one (**4.29**) as a yellow oil: yield 50 mg (64%); silica gel TLC R_f 0.60 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.82-0.85 (m, 3H), 1.21-1.29 (m, 28H), 2.33 (s, 3H), 2.39 (s, 3H) and 2.46-2.52 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 16.0, 17.1, 22.8, 25.8, 28.6, 29.5, 29.75, 29.82, 29.9, 30.1, 32.1, 129.9, 140.3, 143.3, 145.8 and 169.1; mass spectrum (APCI), *m/z* 393.3111 $(M+H)^+$ ($C_{23}H_{41}N_2O_2$ requires *m/z* 393.3117).

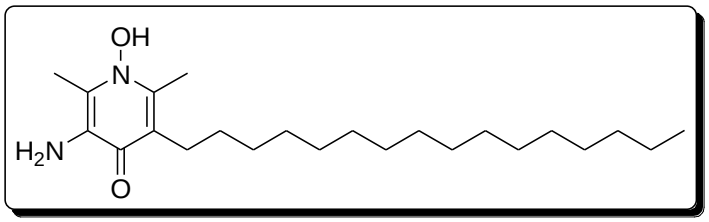


***tert*-Butyl 3-Hexadecyl-2,6-dimethyl-5-nitropyridin-4-yl carbonate (4.30).** To a stirred solution containing 31.4 mg (0.08 mmol) of nitro derivative **4.29** in 3 mL of anhydrous THF was added 13.1 mg (0.113 mmol) of *t*-BuOK and the reaction mixture was stirred at room temperature for 1 h. Then 20.0 mg (0.09 mmol) of Boc_2O was added and the reaction mixtre was heated at 60 °C for 5 h. The cooled reaction mixture was concentrated under diminished pressure to afford a yellow oil. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 hexane–ethyl acetate gave *tert*-butyl 3-hexadecyl-2,6-dimethyl-5-nitropyridin-4-yl carbonate (**4.30**) as

a light yellow oil: yield 17.0 mg (44%); silica gel TLC R_f 0.85 (1:1 hexane–ethyl acetate); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.85-0.89 (m, 3H), 1.25-1.51 (m, 28H), 1.55 (s, 9H) and 2.54-2.58 (s, 8H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 21.5, 22.8, 23.0, 26.7, 27.6, 28.7, 29.4, 29.5, 29.7, 29.77, 29.80, 29.83, 29.9, 32.1, 85.8, 128.9, 139.4, 148.2, 149.5, 149.6 and 161.5; mass spectrum (APCI), *m/z* 493.3652 $(M+H)^+$ ($C_{28}H_{49}N_2O_5$ requires *m/z* 493.3641).



***tert*-Butyl 3-Hexadecyl-2,6-dimethyl-5-nitropyridin-*N*-oxide-4-yl carbonate (4.31).** To a stirred solution containing 27.0 mg (0.05 mmol) of compound **4.30** in 2 mL of anhydrous CH_2Cl_2 was added 10.1 mg (0.06 mmol) of mCPBA. The reaction mixture was stirred at room temperature for 5 h under argon atmosphere, then concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 10:1 ethyl acetate–methanol gave *tert*-butyl 3-hexadecyl-2,6-dimethyl-5-nitropyridin-*N*-oxide-4-yl carbonate (**4.31**) as a light brown solid: yield 18.6 mg (70%); silica gel TLC R_f 0.50 (10:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.83-0.86 (m, 3H), 1.22-1.49 (m, 28H), 1.52 (s, 9H), 2.54 (s, 3H), 2.57 (s, 3H) and 2.60-2.61 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.8, 22.5, 22.9, 23.5, 27.7, 27.9, 28.6, 29.2, 29.4, 29.5, 29.6, 29.7, 29.8, 29.9, 32.2, 86.8, 129.9, 139.8, 148.8, 149.7, 149.9 and 164.5; mass spectrum (APCI), *m/z* 509.3580 $(M+H)^+$ ($C_{28}H_{49}N_2O_6$ requires *m/z* 509.3585).



3-Amino-5-hexadecyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (4.12). To a stirred solution containing 20.0 mg (0.03 mmol) of **4.31** in 2 mL of anhydrous CH_2Cl_2 was added 0.23 mL (342 mg, 3.00 mmol) of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 2 h and then concentrated under diminished pressure. To a solution containing Boc-deprotection product in 2.5 mL of EtOH was added catalytic amount of 10% Pd/C and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a pad of Celite 545® and the filtrate was concentrated under diminished pressure and purified by chromatography on a silica gel column (10 × 1 cm). Elution with 10:1 ethyl acetate–methanol gave 3-amino-5-hexadecyl-1-hydroxy-2,6-dimethylpyridin-4(1*H*)-one (**4.12**) as a light yellow oil: yield 6.0 mg (39% in two steps); silica gel TLC R_f 0.35 (1:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.86-0.89 (m, 3H), 1.25-1.45 (m, 28H), 2.30 (s, 3H), 2.50 (s, 3H) and 2.54-2.57 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 15.9, 18.8, 22.3, 22.6, 25.3, 27.9, 29.3, 29.2, 29.7, 30.0, 30.9, 113.4, 127.1, 145.2, 147.8 and 165.1; mass spectrum (APCI), *m/z* 379.3318 $(M+H)^+$ ($C_{23}H_{43}N_2O_2$ requires *m/z* 379.3325).

REFERENCES

1. Green, R.; Noller, H. F. *Annu. Rev. Biochem.* **1997**, *66*, 679.

2. Wimberly, B. T.; Brodersen, D. E.; Clemons, W. M. Jr.; Morgan-Warren, R. J., Carter, A. P.; Vonrhein, C.; Hartsch, T.; Ramakrishana, V. *Nature* **2000**, *407*, 327.

3. Schmeing, T. M.; Ramakrishnan, V. *Nature* **2009**, *461*, 1234.

4. Karp, G. *Cell and Molecular Biology: Concepts and Experiments* **2002**.

5. Wang, B.; Zhou, J.; Lodder, M.; Anderson, R. D., 3rd; Hecht, S. M. *J. Biol. Chem.* **2006**, *281*, 13865.

6. Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182.

7. Hecht, S. M. *Acc. Chem. Res.* **1992**, *25*, 545.

8. England, P. M. *Biochemistry* **2004**, *43*, 11623.

9. Hendrickson, T. L.; de Crécy-Lagard, V.; Schimmel, P. *Annu. Rev. Biochem.* **2004**, *73*, 147.

10. Zhang, Z. W.; Alfonta, L.; Tian, F.; Busulaya, B.; Uryu, S.; King, D. S.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 8882.

11. Kohrer, C.; Xie, L.; Kellerer, S.; Varshney, U.; RajBhandary, U. L. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14310.

12. Hecht, S. M.; Alford, B. L.; Kuroda, Y.; Kitano, S. *J. Biol. Chem.* **1978**, *253*, 4517.

13. Heckler, T. G.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *J. Biol. Chem.***1983**, *258*, 4492.

14. Heckler, T. G.; Chang, L. H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1984**, *23*, 1468.

15. Robertson, S. A.; Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Nucleic Acids Res.* **1989**, *17*, 9649.

16. Noren, C. J.; Anthony-Cahill, S. J.; Suich, D. J.; Noren, K. A.; Griffith, M. C.; Schultz, P. G. *Nucleic Acids Res.* **1990**, *18*, 83.

17. Dedkova, L. M.; Fahmi, N. E.; Golovine, S. Y.; Hecht, S. M. *J. Am. Chem. Soc.* **2003**, *125*, 6616.

18. Dedkova, L. M.; Fahmi, N. E.; Golovine, S. Y.; Hecht, S. M. *Biochemistry* **2006**, *45*, 15541.

19. Dedkova, L. M.; Fahmi, N. E.; Paul, R.; del Rosario, M.; Zhang, L.; Chen, S.; Feder, G.; Hecht, S. M. *Biochemistry* **2012**, *51*, 401.

20. Maini, R.; Nguyen, D. T.; Chen, S.; Dedkova, L. M.; Chowdhury, S. R.; Alcala-Torano, R.; Hecht, S. M. *Bioorg. Med. Chem.* **2013**, *21*, 1088.

21. Roy Chowdhury, S.; Maini, R.; Dedkova, L. M.; Hecht, S. M. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 4715.

22. Nathans, D.; Neidle, A. *Nature* **1963**, *197*, 1076.

23. Pestka, S. *Annu. Rev. Microbiol.* **1971**, *25*, 487.

24. Hohsaka, T.; Sato, K.; Sisido, M.; Takai, K.; Yokoyama, S. *FEBS Lett.* **1993**, *335*, 47.

25. Starck, S. R.; Qi, X.; Olsen, B. N.; Roberts, R. W. *J. Am. Chem. Soc.* **2003**, *125*, 8090.

26. Ghisaidoobe, A. B.; Chung, S. J. *Int. J. Mol. Sci.* **2014**, *15*, 22518.

27. Katritzky, A. R.; Narindoshvili, T. *Org. Biomol. Chem.* **2009**, *7*, 627.

28. Cornish, V. W.; Benson, D. R.; Altenbach, C. A.; Hideg, K.; Hubbell, W. L.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2910.

29. Mendel, D.; Cornish, V. W.; Schultz, P. G. *Annu. Rev. Biophys. Biomol. Struct.* **1995**, *24*, 435.

30. Hohsaka, T.; Kajihara, D.; Ashizuka, Y.; Murakami, H.; Sisido, M. *J. Am. Chem. Soc.* **1999**, *121*, 34.

31. Hohsaka, T.; Muranaka, N.; Komiyama, C.; Matsui, K.; Takaura, S.; Abe, R.; Murakami, H.; Sisido, M. *FEBS Lett.* **2004**, *560*, 173.

32. Hamada, H.; Kameshima, N.; Szymanska, A.; Wegner, K.; Łankiewicz, L.; Shinohara, H.; Taki, M.; Sisido, M. *Bioorg. Med. Chem.* **2005**, *13*, 3379.

33. Kim, Y.; Ho, S. O.; Gassman, N. R.; Korlann, Y.; Landorf E. V.; Collart, F. R; Weiss, S. *Bioconjug Chem.* **2008**, *3*, 786.

34. Tsien, R. Y. *Annu. Rev. Biochem.* **1998**, *67*, 509.

35. Selvin, P. R. *Nat. Struct. Biol.* **2000**, *7*, 730.

36. Phillips, S. R.; Wilson, L. J.; Borkman, R. F. *Curr. Eye Res.* **1986**, *5*, 611.

37. Zhang, P.; Beck, T.; Tan, W. *Angew. Chem., Int. Ed.* **2001**, *40*, 402.

38. Marti, A. A.; Jockusch, S.; Li, Z.; Ju, J.; Turro, N. J. *Nucleic Acids Res.* **2006**, *34*, e50.

39. Jockusch, S.; Marti, A. A.; Turro, N. J.; Li, Z.; Li, X.; Ju, J.; Stevens, N.; Akins, D. L. *Photochem. Photobiol. Sci.* **2006**, *5*, 493.

40. Miyawaki, A.; Llopis, J.; Heim, R.; McCaffery, J. M.; Adams, J. A.; Ikura, M.; Tsien, R.Y. *Nature* **1997**, *388*, 882.

41. Suzuki, Y.; Yasunaga, T.; Ohkura, R.; Wakabayashi, T.; Sutoh, K. *Nature* **1998**, *396*, 380.

42. Tsien, R. Y.; Miyawaki, A. *Science* **1998**, *280*, 1954.

43. Zhang, Z.; Rajagopalan, P. T.; Selzer, T.; Benkovic, S. J.; Hammes, G. G. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 2764.

44. Antikainen, N. M.; Smiley, R. D.; Benkovic, S. J.; Hammes, G. G. *Biochemistry* **2005**, *44*, 16835.

45. Anderson, R. D., III; Zhou, J.; Hecht, S. M. *J. Am. Chem. Soc.* **2002**, *124*, 9674.

46. Kajihara, D.; Abe, R.; Iijima, I.; Komiyama, C.; Sisido, M.; Hohsaka, T. *Nat. Methods* **2006**, *3*, 923.

47. Chen, S.; Fahmi, N. E.; Wang, L.; Bhattacharya, C.; Benkovic, S. J.; Hecht, S. M. *J. Am. Chem. Soc.* **2013**, *135*, 12924.

48. Chen, S.; Fahmi, N. E.; Bhattacharya, C.; Wang, L.; Jin, Y.; Benkovic, S. J.; Hecht, S. M. *Biochemistry* **2013**, *52*, 8580.

49. Dey, B.; Thukral, S.; Krishnan, S.; Chakrobarty, M.; Gupta, S.; Manghani, C.; Rani, V. *Mol. Cell. Biochem.* **2012**, *65*, 279.

50. Travers, A. DNA–Protein Interactions. **1993**, London: Chapman and Hall.

51. Pabo, C. O.; Sauer, R. T. *Annu. Rev. Biochem.* **1984**, *53*, 293.

52. Dickerson, R. E. *Sci. Am.* **1983**, *249*, 94.

53. Luscombe, N.; Austin, S.E.; Berman, H. M.; Thornton, J.M. *Genome Biol.* **2000**, *1*, 1.

54. Strauch, M. A. Protein–DNA Complexes: Specific. **2001**, Encyclopedia of Life Sciences.

55. Pavletich, N.P.; Pabo, C.O. *Science* **1991**, *252*, 809.

56. Harrison, S. C.; Aggarwal, A. K. *Annu. Rev. Biochem.* **1990**, *59*, 933.

57. Stuiver, M. H.; van der Vliet, P. C. *J. Virol.* **1990**, *64*, 379.

58. Nadassy, K.; Wodak, S. J.; Janin, J. *Biochemistry* **1999**, *38*, 1999.

59. Xiong, Y.; Sundaralingam, M. Protein-nucleic acid interaction: major groove recognition determinants. In *Encyclopedia of Life Sciences*. **2001**, London: Macmlllan Reference Ltd.

60. Kielkopf, C. L.; White, S.; Szewczyk, J. W. *Science* **1998**, *282*, 111.

61. Schwabe, J. W. R. *Current Opinion in Structural Biology* **1997**, *7*, 126.

62. Biot, C.; Buisine. E; Kwasigroch, J. M.; Wintjens, R.; Rooman, M. *J. Biol. Chem.* **2002**, *277*, 40816.

63. Nelson, D. L.; Cox, M. M. *Lehninger Principles 6th ed.* **2012**, New York: Freeman, W. H. & Company.

64. Henze, K.; Martin, W. *Nature* **2003**, *426*, 127.

65. Saraste, M. W. *Science* **1999**, *283*, 1488.

66. Newmeyer, D. D.; Ferguson-Miller, S. *Cell* **2003**, *112*, 481.

67. Stryer, L. *Biochemistry 4th ed.* **1995**, New York: Freeman, W. H. & Company.

68. Hinkle, P.C.; Kumar, M. A.; Resetar, A.; Harris, D.L. *Biochemistry* **1991**, *30*, 3576.

69. Nelson, D. L.; Cox, M. M. *Lehninger Principles 3rd ed.* **2000**, New York: Freeman, W. H. & Company.

70. Voet, D.; Voet, J.; Pratt, C.W. *Fundamentals of Biochemistry* **1999**, New York: John Wiley & Sons, Inc.

71. Guerra, G.; Martinez, F.; Pardo, J.P. *Biochem. Mol. Biol. Educ.* **2002**, *30*, 363.

72. Galkin, A. S.; Grivennikova, V. G.; Vinogradov, A. D. *FEBS Lett.* **1999**, *451*, 157.

73. Galkin, A.; Dröse, S.; Brandt, U. *Biochim. Biophys. Acta* **2006**, *1757*, 1575.

74. Turrens, J. F. *J. Physiol*. **2003**, *552*, 335.

75. Murphy, M. P. *Biochem. J*. **2009**, *417*, 1.

76. Conklin, K.A. *Integ. Cancer Ther.* **2004**, *3*, 294.

77. Yokoyama, Y.; Beckman, J. S.; Beckman, T. K.; Wheat, J. K.; Cash, T. G.; Freeman, B. A.; Parks, D. A. *Am. J. Physiol.* **1990**, *258*, G564.

78. Liochev, S. I.; Fridovich, I. *IUBMB Life*, **1999**, *48*, 157.

79. Fenton, H. J. H. *J. Chem. Soc.* **1894**, *65*, 899.

80. Zhang, Y.; Marcillat, O.; Giolivi, C.; Ernster, L.; Davies, D. J. A. *J. Biol.Chem.* **1990**, *265*, 16330.

81. Mates, J. M.; Parez-Gomez, C.; Nunez de Castro, I. *Clin. Biochem.* **1999**, *32*, 595.

82. Fridovich, I. *Ann. N. Y. Acad. Sci.* **1999**, *893*, 13.

83. Benzie, I. F. F. *Eur. J. Nut.* **2000**, *39*, 53.

84. Santos, D.; Palmeira, C.; Seica, R.; Dias, J.; Mesquita, J.; Moreno, A.; Santos, M. *Mol. Cell. Biochem.* **2003**, *246*, 163.

85. Battisti, C.; Formichi, P.; Cardaioli, E.; Bianchi, S.; Mangiavacchi, P.; Tripodi, S.; Tosi, P.; Federico, A. *J. Neurol. Neurosurg. Psych.* **2004**, *75*, 1731.

86. Emerit, J.; Edeas, M.; Bricaire, F. *Biomed. Pharmacother.* **2004**, *58*, 39.

87. Lin, M. T.; Beal, M. F. *Nature* **2006**, *443*, 787.

88. Pieczenik, S. R.; Neustadt, J. *Exp. Mol. Path.* **2007**, *83*, 84.

89. Friederich, M.; Hansell, P.; Palm, F. *Curr. Diabetes Rev.* **2009**, *5*, 120.

90. Fernandez-Checa, J.; Fernandez, A.; Morales, A.; Mari, M.; Garcia-Ruiz, C.; Colell, A. *CNS Neurol. Disord. Drug Targets* **2010**, *9*, 439.

91. Rocha, M.; Apostolova, N.; Hernandez-Mijares, A.; Herance, R.; Victor, V. *Curr. Med. Chem.* **2010**, *17*, 3827.

92. Aksenov, M. Y.; Tucker, H. M.; Nair, P.; Aksenova, M. V.; Butterfield, D. A.; Estus, S.; Markesbery, W. R. *Neurochem. Res.* **1999**, *24*, 767.

93. Kulkarni, R.; Marples, B.; Balasubramaniam, M.; Thomas, R. A.; Tucker, J. D. *Radiation Res.* **2010**, *173*, 635.

94. Armstrong, J. S.; Khdour, O.; Hecht, S. M. *FASEB J.* **2010**, *24*, 2152.

95. DiMauro, S.; Schon, E. A. *Annu. Rev. Neurosci.* **2008**, *31*, 91.

96. McLellan, M. E.; Kajdasz, S. T.; Hyman, B. T.; Bacskai, B. J. *J. Neurosci.* **2003**, *23*, 2212.

97. Ikebe, S.-i.; Tanaka, M.; Ohno, K.; Sato, W.; Hattori, K.; Kondo, T.; Mizuno, Y.; Ozawa, T. *Biochem. Biophys. Res. Commun.* **1990**, *170*, 1044.

98. Beal, M. F. *Ann. N. Y. Acad. Sci.* **2003**, *991*, 120.

99. Höglinger, G. U.; Carrard, G.; Michel, P. P.; Medja, F.; Lombès, A.; Ruberg, M.; Friguet, B.; Hirsch, E. C. *J. Neurochem.* **2003**, *86*, 1297.

100. Wiedemann, F. R.; Manfredi, G.; Mawrin, C.; Beal, M. F.; Schon, E. A. *J. Neurochem.* **2002**, *80*, 616.

101. Esterbauer, H.; Schaur, R. J.; Zollner, H. *Free Rad. Biol. Med.* **1991**, *11*, 81.

102. Young, I. S.; McEneny, J. *Biochem. Soc. Trans.* **2001**, *29*, 358.

103. Porter, N. A. *Acc. Chem. Res.* **1986**, *19*, 262.

104. Mellors, A.; Tappel, A. L. *J. Biol. Chem.* **1966**, *241*, 4353.

105. Takayanagi, R.; Takeshige, K.; Minakami, S. *Biochem. J.* **1980**, *192*, 853.

106. Ernster, L. D.; *Biochim. Biophys. Acta.* **1995**, *1271*, 195.

107. Burton, G. W.; Ingold, K. U. *Acc. Chem. Res.* **1986**, *19*, 194.

108. King, M. S.; Sharpley, M. S.; Hirst, J. *Biochemistry* **2009**, *48*, 2053.

109. Esposti, M. D.; Linnane, A. W.; Ghelli, A.; Ngo, A.; Helfenbaum, L. *J. Bioenerg. Biomembr.* **1997**, *29*, 71.

110. Takenaka, Y.; Miki, M.; Yasuda, H.; Mino, M. *Arch. Biochem. Biophys.* **1991**, *285*, 344.

111. Kamal-Eldin, A.; Appelqvist, L. *Lipids* **1996**, *31*, 671.

112. Brigelius-Flohe, R.; Traber, M. G. *J. Biol. Chem.* **2004**, *279*, 39414.

113. Tallman, K.; Pratt, D. A.; Porter, N. *J. Am. Chem. Soc.* **2001**, *123*, 11827.

114. Fennema, O. R. *Food Chemistry*, 3rd ed.; **1996**, CRC Press: New York.

115. Maillard, B.; Ingold, K. U.; Scaiano, J. C. *J. Am. Chem. Soc.* **1983**, *105*, 5095.

116. Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6472.

117. Lowes, D.; Thottakam, B.; Webster, N.; Murphy, M.; Galley, H. *Free Rad. Biol. Med.* **2008**, *45*, 1559.

118. Pisano, P. *Eur. J. Pharmacol.* **1996**, *51*, 167.

119. Fujisawa, S.; Kadoma, Y.; Yokoe, I. *Chem Phys Lipids.* **2004**, *130*, 189.

120. Merkel, L.; Hoesl M.G.; Albrecht M.; Schmidt A.; Budisa N. *Chembiochem.* **2010**, *11*, 305.

121. Lepthien, S.; Hoesl, M. G.; Merkel, L.; Budisa, N. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 16095.

122. Chen, Y.; Rich, R. L.; Gai, F.; Petrich, J. W. *J. Phys. Chem.* **1993**, 97, 1770.

123. Tucker, M. J.; Oyola, R.; Gai, F. *Biopolymers* **2006**, *83*, 571.

124. Taskent-Sezgin, H.; Marek, P.; Thomas, R.; Goldberg, D.; Chung, J.; Carrico, I.; Raleigh D. P. *Biochemistry* **2010**, *49*, 6290.

125. Waegele, M. M.; Tucker, M. J.; Gai, F. *Chem. Phys. Lett.* **2009**, *478*, 249.

126. Schollkopf, U.; Groth, U.; Westphalen, K.; Deng, C. *Synthesis* **1981**, 969.

127. Miller, G. P.; Benkovic, S. J. *Chem. Biol.* **1998**, *5*, R105.

128. Szymanska, A.; Wegner, K.; Lankiewicz, L. *Helv. Chim. Acta.* **2003**, *86*, 3326.

129. Speight, L.C.; Muthusamy, A. K.; Goldberg, J. M.; Warner, J. B.; Wissner, R. F.; Willi, T. S.; Woodman, B. F.; Mehl, R. A.; Petersson, E. J. *J. Am. Chem. Soc.* **2013**, *135*, 18806.

130. Wang, Z.; Talukder, P.; Hecht, S.M.; Chen, S. *Bioorg Med Chem Lett.* **2015**, *25*, 1182.

131. Wei-Guo, S.; Hong, J.; Guangxiu, D. WO 2011079804 A1, Jul 07, 2011.

132. Yao, C. H.; Song, J. S.; Chen, C. T.; Yeh. T. K.; Hsieh, T. C.; Wu, S. H.; Huang, C. Y.; Huang, Y. L.; Wang, M. H.; Liu, Y. W.; Tsai, C. H.; Kumar, C. R.; Lee, J. C. *Eur. J. Med. Chem.* **2012**, *55*, 32.

133. Ramakrishna, V. S. N.; Shirsath. V. S.; Kambhampati, R. S.; Rao, V. S. V. V.; Jasti. V. WO 2004048330 A1, Jun 10, 2004.

134. Bajwa, J. S.; Chen, G.; Prasad, K.; Repic, O.; Blacklock, T. J. *Tetrahedron Lett.* **2006**, *47*, 6425.

135. Briere, J.F.; Dupas, G.; Queguiner, G.; Bourguignon, J. *Heterocycles* **2000**, *52*, 1371.

136. Lang, F.; Zewge, D.; Houpis, I. N.; Volante, R. P. *Tetrahedron Lett*. **2001**, *42*, 3251.

137. Sy, W. *Syn. Commun.* **1992**, *22*, 3215.

138. Zheng, J.; Deng, L.; Chen, M.; Xiao, X.; Xiao, S.; Guo, C.; Xiao, G.; Bai, L.; Ye, W.; Zhang, D.; Chen, H. *Eur. J. Med. Chem.* **2013**, *65*, 158.

139. Ballet, S.; Feytens, D.; Buysse, K.; Chung, N. N; Lemieux, C.; Tumati, S.; Keresztes, A.; Van Duppen, J.; Lai, J.; Varga, E.; Porreca, F.; Schiller, P. W; Vanden Broeck, J.; Tourwé, D. *J Med Chem.* **2011**, *54*, 2467.

140. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed., 2006, Springer, New York.

141. Karginov, V. A.; Mamaev, S. V.; An, H.; Van Cleve, M. D.; Hecht, S. M.; Komatsoulis, G. A.; Abelson, J. N. *J. Am. Chem. Soc.* **1997**, *119*, 8166.

142. Patchornik, A.; Amit, B.; Woodward, R. B. *J. Am. Chem. Soc.* **1970**, *92*, 6333.

143. Talukder, P.; Chen, S.; Liu, C. T.; Baldwin, E. A.; Benkovic, S. J.; Hecht S. M. *Bioorg. Med. Chem.* **2014**, *22*, 5924.

144. Bolin, J. T.; Filman, D. J.; Matthews, D. A.; Hamlin, R. C.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 13650.

145. Talukder. P.; Chen, S.; Roy, B.; Yakovchuk, P.; Spiering, M. M.; Alam, M. P.; Madathil, M. M.; Bhattacharya, C; Benkovic, S. J.; Hecht, S. M. *Biochemistry*

2015, *54*, 7457.

146. Fierke, C. A.; Johnson, K. A.; Benkovic, S. J. *Biochemistry* **1987**, *26*, 4085.

147. Bernabeu, M.C.; Díaz, J.L.; Jiménez, O.; Lavilla, R. *Synth. Commun.* **2004**, *34*, 137.

148. Bystroff, C.; Oatley, S. J.; Kraut, J. *Biochemistry* **1990**, *29*, 3263.

149. Ray, A.; Nordén, B. *FASEB J*. **2000**, *9*, 1041.

150. Matsumura, S.; Takahashi, T.; Ueno, A.; Mihara, H. *Chem. Eur. J.* **2003**, *9*, 4829.

151. Takahashi, T.; Hamasaki, K.; Ueno, A.; Mihara, H. *Bioorg. Med. Chem*. **2001**, *9*, 991.

152. Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497.
153. Thiede, C.; Bayerdorffer, E.; Blasczyk, R.; Wittig, B.; Neubauer, A. *Nucleic Acids Res.* **1996**, *24*, 983.

154. Wang, J.; Palecek, E.; Nielsen, P. E.; Rivas, G.; Cai, X.; Shirashi, H.; Dontha, N.; Luo, D.; Farias, P. A. M. *J. Am. Chem. Soc.* **1996**, 118, 7667.

155. Demidov, V.; Frank-Kamenetskii, M. D.; Egholm, M.; Buchardt, O.; Nielsen, P. E. *Nucleic Acids Res*. **1993**, *21*, 2103.

156. Demers, D. B.; Curry, E. T.; Egholm, M.; Sozer, A. C. *Nucleic Acids Res.* **1995**, *23*, 3050.

157. Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Anti-Cancer Drug Design* **1993**, *8*, 53.

158. Boffa, L. C.; Morris, P. L.; Carpeneto, E. M.; Louissaint, M.; Allfrey, V. G. *J. Biol. Chem.* **1996**, *271*, 13228.

159. Miyanishi, H.; Takahashi, T.; Mihara, H. *Bioconjugate Chem*. **2004**, *15*, 694.

160. Takahashi, T.; Yana, D.; Mihara, H. *Chem.Biochem*. **2006**, *7*, 729.

161. Watanabe, S.; Tomizaki, K.; Takahashi, T.; Usui, K.; Kajikawa, K.; Mihara, H. *Peptide Sci.* **2007**, *88*, 131.

162. Roviello, G. N.; Gröschel, S.; Pedone, C; Diederichsen, U. *Amino Acids* **2010**, *38*, 1301.

163. Kang, H. J.; Kendrick, S.; Hecht, S. M.; Hurley, L. H. *J. Am. Chem. Soc.* **2014**, *136*, 4172.

164. Zhang, Y. *BMC Bioinformatics* **2008**, *9*, 40.

165. Peifer, M.; Giacomo, F. D.; Schandl, M.; Vasella, A. *Helv. Chim. Acta* **2009**, *92*, 1134.

166. Preus, S.; Kilså K.; Wilhelmsson, L. M.; Albinsson, B. *Phys. Chem. Chem. Phys.* **2010**, *12*, 8881.

167. Sandin, P.; Lincoln, P.; Brown, T.; Wilhelmsson, L. M. *Nat. Protoc.* **2007**, *2*, 615.

168. Maini, R.; Dedkova, L. M.; Paul, R.; Madathil, M. M.; Chowdhury, S. R.; Chen, S.; Hecht, S. M. *J. Am. Chem. Soc.* **2015**, *137*, 11206.

169. Hellman, L. M.; Fried, M. G. *Nat. Prot*. **2007**, *2*, 1849.

170. Turunen, M.; Olsson, J.; Dallner, G. *Biochim. Biophys. Acta* **2004**, *1660*, 171.

171. Bentinger, M.; Brismar, K.; Dallner, G. *Mitochondrion* **2007**, *7*, S41.

172. Aberg, F.; Appelkvist, E. L.; Dallner, G.; Ernster, L. *Arch. Biochem. Biophys.* **1992**, *295*, 230.

173. Mellors, A.; Tappel, A. L. *J. Biol. Chem.* **1966**, *241*, 4353.

174. Lynch, D. R.; Perlman, S. L.; Meier, T. *Arch. Neourol.* **2010**, *67*, 941.

175. Meier, T.; Perlman, S. L.; Rummey, C.; Coppard, N. J.; Lynch, D. R. *J. Neurol.* **2012**, *259*, 284.

176. Weidemann, F.; Rummey, C.; Bijnens, B.; Störk, S.; Jasaityte, R.; Dhooge, J.; Baltabaeva, A.; Sutherland, G.; Schulz, J. B.; Meier, T. *Circulation* **2012**, *125*, 1626.

177. Metz, G.; Coppard, N.; Cooper, J. M.; Delatycki, M. B.; Dürr, A.; Prospero, N. A. D.; Giunti, P.; Lynch, D. R.; Schulz, J. B.; Rummey, C.; Meier, T. *Brain* **2013**, *136*, 259.

178. Fato, R.; Bergamini, C.; Leoni, S.; Lenaz, G. *BioFactors* **2008**, *32*, 31.

179. Esposti, M. D.; Ngo, A.; Ghelli, A.; Benelli, B.; Carelli, V.; McLennan, H.; Linnane, A. W. *Arch. Biochem. Biophys.* **1996**, *330*, 395.

180. Brière, J. J.; Schlemmer, D.; Chretien, D.; Rustin, P. *Biochem. Biophys. Res. Commun.* **2004**, *316*, 1138.

181. Pratt, D. A.; DiLabio, G. A.; Brigati, G.; Pedulli, G. F.; Valgimigli, L. *J. Am. Chem. Soc.* **2001**, *123*, 4625.

182. Valgimigli, L.; Brigati, G.; Pedulli, G. F.; DiLabio, G. A.; Mastragostino, M.; Arbizzani, C.; Pratt, D. A. *Chem. Eur. J.* **2003**, *9*, 4997.

183. Wijtmans, M.; Pratt, D. A.; Valgimigli, L.; DiLabio, G. A.; Pedulli, G. F.; Porter, N. A. *Angew. Chem. Int. Ed.* **2003**, *42*, 4370.

184. Arce, P. M.; Khdour, O. M.; Goldschmidt, R.; Armstrong, J. S.; Hecht, S. M. *ACS Med. Chem. Lett.* **2011**, *2*, 608.

185. Arce, P. M.; Goldschmidt, R.; Khdour, O. M.; Madathil, M. M.; Jaruvangsanti, J.; Dey, S.; Fash, D. M.; Armstrong, J. S.; Hecht, S. M. *Bioorg. Med. Chem.* **2012**, *20*, 5188.

186. Goldschmidt, R.; Arce, P. M.; Khdour, O. M.; Collin, V. C.; Dey, S.; Jaruvangsanti, J.; Fash, D. M.; Hecht, S. M. *Bioorg. Med. Chem.* **2013**, *21*, 969.

187. Khdour, O. M.; Arce, P. M.; Roy, B.; Hecht, S. M. *ACS Med. Chem. Lett.* **2013**, *4*, 724.

188. Leiris, S. J.; Khdour, O. M.; Segerman, Z. J.; Tsosie, K. S; Chapuis, J.; Hecht, S. M. *Bioorg. Med. Chem.* **2010**, *18*, 3481.

189. Yeates, C. L.; Batchelor, J. F.; Capon, E. C.; *J. Med. Chem.* **2008**, *51*, 2845.

190. Venkataraman, S.; Barange, D. K.; Pal, M. *Tetrahedron Lett.* **2006**, *47*, 7317.

191. Woschek, A.; Mahout, M.; Mereiter, K.; Hammerschmidt, F. *Synthesis* **2007**, *10*, 1517.

192. Kitagawa, H.; Kumura, K.; Takahata, S.; Iida, M.; Atsumi, K. *Bioorg. Med. Chem.* **2007**, *15*, 1106.

193. Bradbury R. H.; Allott. C. P.; Dennis, M.; Girdwood, J. A.; Kenny, P.W.; Major J. S. ; Oldham, A. A.; Ratcliffe, A. H.; Rivett, J. E.; Roberts, D. A. *J. Med. Chem.* **1993**, *36*, 1245.

194. Tietze, L. F; Ma, L. *Heterocycles* **2010**, *82*, 377.

195. Burton, A. G.; Halls, P. J.; Katritzky, A. R. *J. Perkin Trans II* **1972**, *2*, 1953.

APPENDIX A

COPYRIGHT PERMISSION

A)

Licensee: Poulami Talukder.

License Date: Nov 1, 2014.

License Number: 3840050495893.

Publication: Bioorganic & Medicinal Chemistry.

Title: Small Molecules as Probes of Biological Systems.

Type of Use: reuse in a thesis/dissertation.

B)

"Reprinted with permission from (Talukder. P.; Chen, S.; Roy, B.; Yakovchuk, P.; Spiering, M. M.; Alam, M. P.;Madathil, M. M.; Bhattacharya, C; Benkovic, S. J.; Hecht, S. M. *Biochemistry* **2015**, *54*, 7457). Copyright (2015) American Chemical Society."